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African Trypanosome Genes Specifically Required for Fitness in vivo

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*Award date:*  
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AFRICAN TRYPANOSOME GENES  
SPECIFICALLY REQUIRED FOR FITNESS  
*IN VIVO*

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MSC BY RESEARCH  
APRIL 2017



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## ACKNOWLEDGEMENTS

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I want to thank Professor David Horn for giving me the opportunity to work on this interesting and challenging project and for his support and supervision throughout. I also want to thank Lucy Glover for her technical assistance, and the rest of David Horn's lab for giving me a warm welcome and for always being ready to answer any questions or provide any help needed.

## DECLARATION

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I declare that the content of this project report is my own work and has not previously been submitted for any other assessment. The report is written in my own words and conforms to the University of Dundee's Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report.

James Shrimpton, 14/07/2017



## SUMMARY

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This report details the initial validation of hits from a genome-scale RNAi fitness screen to reveal genes that showed a specific loss of fitness *in vivo*. Analyses of African trypanosome biology are often carried out in HMI culture medium which is rich in nutrients and may not necessarily be representative of the environments encountered *in vivo*. We reasoned that parasites *in vivo* are likely to display increased dependence upon certain nutritional pathways as well as defences against oxidative stress and innate immune attack.

Prior to this project, the lab ran a genome-scale RNAi fitness-profiling screen in rats in order to address this question. Analysis of the resulting list of genes that displayed a specific loss-of-fitness *in vivo* showed two genes linked to phosphatidylinositol metabolism, providing validation for the screen. Other hits included genes linked to folate metabolism and to DNA repair.

This report details the initial analysis and validation of the hits from the screen and the development of systems for more detailed follow-up. One particularly promising approach described here is the growth of *T. brucei* in a simple serum system, which we believe effectively mimics aspects of the *in vivo* environment. Two genes were successfully validated, one of which has since been independently confirmed in the literature, and initial investigations into the underlying biology behind their phenotypes are described. The appendices to this report provide further detail regarding the hits from the *in vivo* screen, as well as describing two other projects which I worked on during my time in David Horn's laboratory.

## LIST OF ABBREVIATIONS

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5-MetE: 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase

5-MTHF: 5-methyltetrahydrofolate

AAT: Animal African Trypanosomiasis

APO-L1: apolipoprotein L1

BLAST: Basic Local Alignment Search Tool

CDD: Conserved domain database

CMM: Creek's Minimal Media

CNS: Central Nervous System

DHFR: Dihydrofolate Reductase

FBS: Foetal Bovine Serum

GPI: glycosylphosphatidylinositol

GPI-PLC: glycosylphosphatidylinositol-specific phospholipase C

HAT: Human African Trypanosomiasis

IMDM: Iscove's modification of Dulbecco's Minimal Essential Medium of Eagle

IMP: inositol monophosphatase

MEM: Modified Eagles Medium

RIT-seq: RNAi target sequencing

VEX1: VSG exclusion 1 protein

VSG: Variant Surface Glycoprotein

## INTRODUCTION

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### TRYPANOSOMES AND DISEASE

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#### THE TRYPANOSOMATIDS

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The African trypanosomes belong to a group of kinetoplastid protozoans known as the trypanosomatids. Several trypanosomatids are parasitic, mono-flagellated organisms with a 'corkscrew-like' shape (the name trypanosomatid comes from the Greek meaning 'screw-borer body'<sup>1</sup>). They are a diverse and highly divergent group of eukaryotes with a variety of life cycles in both arthropod vectors and mammalian hosts and include several species which cause human disease<sup>2</sup>. As they are highly divergent from the main eukaryotic lineage, the trypanosomatids also contain a number of unique organelles such as the glycosomes<sup>3,4</sup> and kinetoplasts<sup>5-9</sup>.

There are three key neglected tropical diseases which are caused by trypanosomatid organisms, each spread by various biting insects. Spread by the sand fly, *Leishmania* spp. cause leishmaniasis, which presents in three forms: cutaneous, mucocutaneous and visceral. Of the three, the first shows the greatest morbidity, while the highest mortality is seen in the visceral form<sup>10</sup>. The South American trypanosome, *Trypanosoma cruzi*, causes Chagas disease and is spread by the triatomine bug. The African trypanosome, *Trypanosoma brucei*, is the focus of this project. It causes Human African Trypanosomiasis (HAT) and is spread by the bite of the tsetse fly. Collectively, these diseases cost thousands of lives in some of the poorest parts of the world and cause millions of pounds of economic damage. With existing drugs being

unsatisfactory, even before current trends of emerging resistance, new treatments and diagnostics are desperately needed.

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### THE AFRICAN TRYPANOSOMES

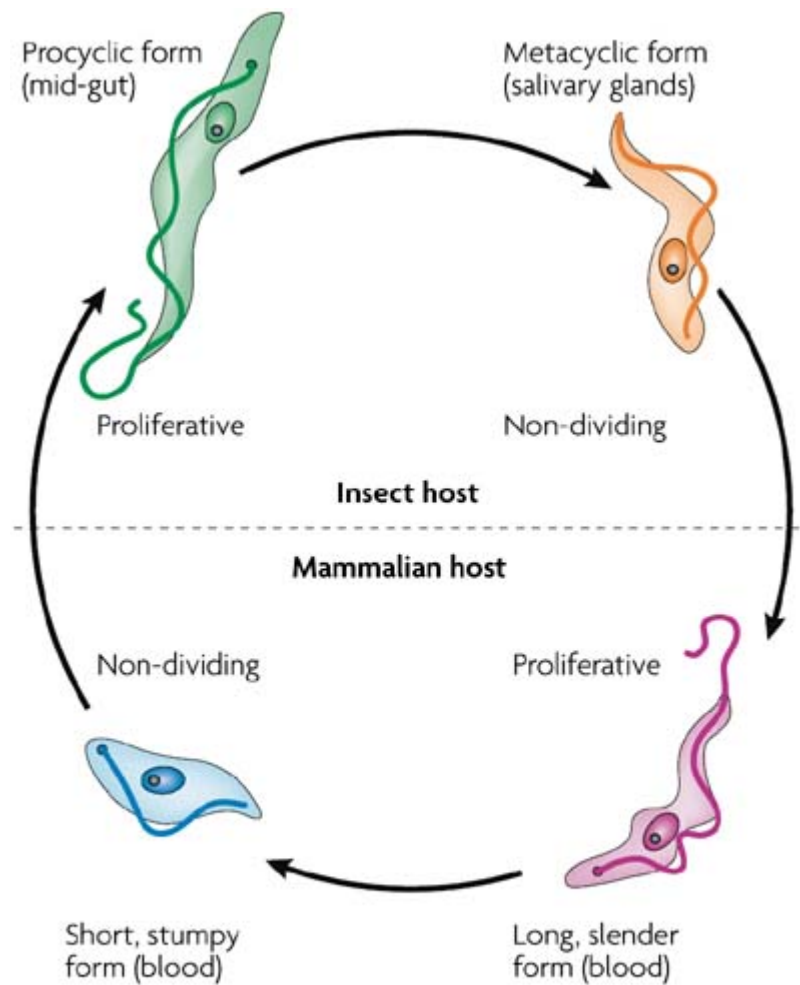
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*Trypanosoma evansi* was the first pathogenic trypanosome to be discovered (in horses and camels) in 1880. Fourteen years later, David Bruce identified *Trypanosoma brucei* as the causative agent of nagana in cattle<sup>11</sup>. Diagnosis of the African trypanosomes as the pathogen behind HAT followed in 1902.

*Trypanosoma brucei* spp. are unicellular, obligate eukaryotic parasites with a complex life-cycle across both insect vectors and mammalian hosts (Figure 1). The procyclic form of the parasite replicates in the midgut of various *Glossina* spp., the tsetse fly. It then migrates to the insect's salivary glands – morphing into the metacyclic form. This form is primed for infection by the generation of the surface coat which protect the parasite from immune-mediated clearance. When the tsetse takes blood from the mammalian host, the parasites are subdermally injected into the host. They proliferate at the site of infection, differentiating into long, slender bloodstream forms which migrate to the vasculature while a population remains in the skin to be taken up in future tsetse blood meals, increasing transmission<sup>12</sup>. From the vasculature, the trypanosomes can then invade other organs, particularly the central nervous system (CNS). The migration of the parasites into the CNS triggers the second stage of the disease. The time elapsing before this migration is dependent on the sub-species of the parasite, taking a few weeks for *T. b. rhodesiense* but an average of a year and a half for *T. b. gambiense*<sup>13,14</sup>. Once the parasite is in the CNS, it causes the neuropsychiatric

symptoms described below that gave the disease its common name: African sleeping sickness.

FIGURE 1: THE *TRYPANOSOMA BRUCEI* LIFE CYCLE



Nature Reviews | Microbiology

The *Trypanosoma brucei* life cycle. A simplified diagram showing *T. brucei* alternating between the insect vector and the mammalian host. Rapidly dividing forms of the trypanosome in each stage eventually differentiate into non-dividing quiescent cells which are adapted for arrival into the human host, in the case of metacyclics, or the insect vector, in the case of 'stumpy form' parasites. Diagram taken from <sup>15</sup>, having been adapted from the original from *Nature Reviews Microbiology* <sup>16</sup> © (2006) Macmillan Publishers Ltd.

Sleeping sickness was first described in the 14<sup>th</sup> century and has taken a heavy toll on both humans and livestock in Africa over the centuries. Between 1896 and 1908, Human African Trypanosomiasis killed approximately one million people in what is now Zaire and the North shore of Lake Victoria. Due to concerted control efforts, and the rise of cheap insecticides such as DDT providing improved tsetse control, this dropped dramatically until in the 1970s it was estimated that the yearly incidence of new infections was only twenty thousand<sup>17</sup>.

However, with the apparent retreat of the disease coinciding with upheaval and unrest in the affected countries, the political will to tackle African sleeping sickness was lost and the disease resurged. By 2001, reports estimated that in some areas within the Democratic Republic of Congo (the focal point for *T. b. gambiense*) the prevalence rate had risen to be in excess of 60% and the World Health Organisation described African trypanosomiasis as “truly a re-emerging infectious disease”<sup>17</sup>. More recent estimates place the number at approximately 300,000 people infected, with sixty million living in at-risk areas. This means a total of anywhere between ten and forty thousand deaths every year<sup>1,18</sup> and it is suspected that these figures are underreported due to the lack of health infrastructure in affected areas and the social stigma associated with the disease<sup>19,20</sup>. Untold amounts of economic damage have also been caused by animal African trypanosomiasis (AAT), also known as Nagana, the cattle disease caused by *T. b. brucei*, which has been recently reviewed by Morrison et al.<sup>21</sup>

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## THE DISEASE: SYMPTOMS

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As noted above, Human African Trypanosomiasis is a two-stage disease. Early illness is associated with symptoms which are non-specific and, as a result, the disease can be challenging to diagnose. These non-specific symptoms include fatigue, headaches, fever, general malaise and enlarged lymph nodes. The second stage has progressively worsening neuropsychiatric symptoms such as sleep disturbance which, if untreated, will eventually lead to coma and death<sup>22</sup>. The disease is considered to have a 100% fatality rate if untreated<sup>1,22</sup> although some studies contradict this (reviewed in <sup>14</sup>) and reports of infection-tolerant individuals continue to emerge.

*T. b. gambiense* causes the most common form of trypanosomiasis, causing over 95% of recorded cases and appearing primarily in West and Central Africa. *T. b. rhodesiense*, which is the less common form, is endemic in East and Southern Africa. The reason for this limited geographical localisation is that, unlike *T. b. gambiense*, *T. b. rhodesiense* is zoonotic and requires an animal reservoir. Currently, the two continue to be largely geographically distinct from each other. However, migration of *T. b. rhodesiense* means that incidences of the two diseases are starting to overlap in Uganda<sup>1,23</sup>. It is also becoming increasingly obvious that there is substantial variation in disease progression even within the subspecies of the parasite, with *T. b. rhodesiense* infections in Uganda progressing much more rapidly than they do in Malawi, for example<sup>24</sup>.

The two forms of the parasite complicate treatment, as *T. b. rhodesiense* is resistant to some of the safer drugs that can be used to treat *T. b. gambiense* HAT. Treatment is additionally complicated by the inability of some drugs to cross the blood-brain barrier.



Further difficulties exist due to the limitations of the existing diagnostic testing methods. The Card Agglutination Test for Trypanosomiasis (CATT) is a serological test which determines whether patient antibodies can bind to and aggregate suspended trypanosome samples<sup>25</sup>. However, due to a number of limitations which have been extensively discussed in the literature, such as being unable to detect *T. b. rhodesiense* HAT, high production costs and the requirement for extensively trained specialist personnel to operate the test, various alternatives have been sought for CATT<sup>22,26–30</sup>. One promising solution is a simple, high-sensitivity, lateral-flow device. Some progress has been made towards developing such a tool. However, it has not yet reached widespread use in the field and would not negate the necessity of a lumbar puncture to determine the stage of the disease<sup>30–32</sup>.

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## THE DISEASE: TREATMENT

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The main treatment of first-stage *T. b. gambiense*, Pentamidine, has remained the same for more than half a century despite the impracticality of delivering the required repeated intravenous infusions in a resource-poor setting<sup>33</sup>. The primary drug for *T. b. rhodesiense*-mediated trypanosomiasis is Suramin, a drug that was discovered in 1917 and has a range of serious side effects such as nausea, severe abdominal pain, diarrhoea and vomiting<sup>34</sup>. Melarsoprol, which superseded other arsenic-based compounds in the 1950s,<sup>35</sup> remains the only drug which can be used to treat late-stage *T. b. rhodesiense*. Unfortunately, the drug also causes encephalopathic syndrome in up to 10% of patients, half of whom die as a result. Positively, however, it is an extremely potent drug and displays an EC50 in the low nanomolar range<sup>36</sup>.

These examples serve to show the huge dearth of successful research into the treatment of trypanosomiasis for much of the last hundred years. However, improvements are slowly being made. Eflornithine is a trypanostatic drug which was originally developed for cancer. Initially trialled alone, it required four daily infusions for two weeks – resulting in a 90% cure rate<sup>37</sup>. However, it has proven to be more effective, with reduced side-effects, when used in combination with nifurtimox, a drug used to treat Chagas disease<sup>38,39</sup>. This combination therapy is now the frontline treatment for late-stage disease caused by *T. b. gambiense*. There are also two drugs in the pipeline for HAT. The more advanced of these is fexinidazole, thought to be a prodrug which is rapidly metabolised *in vivo* to form sulfoxide and sulfone<sup>40</sup>, and which could be sufficient to cure HAT with once-daily doses. However, nifurtimox and fexinidazole have the potential for cross-resistance<sup>41</sup>. This illustrates the danger of

relying on the targeting of a small selection of known pathways and the need to constantly look for untargeted pathways to target with new therapies in order to keep ahead of the rapidly adapting parasite.

To date, there remains no prospect of a successful vaccine, and the various drugs that currently treat the disease are too dangerous to take as prophylactics. As a result, preventative measures are primarily focused on vector control. In the 1960s it looked as if trypanosomiasis could be controlled, if not eliminated. However, decolonisation, war and the loss of political will led to decades of neglect. This resulted in increasing numbers of new cases. The World Health Organisation responded with new efforts to treat and control the disease, new funding was made available and the pharmaceutical industry committed to the continued production of the existing drugs. The disease is now on track for eventual eradication<sup>1</sup>. However, as described above, this situation has arisen before.

The threat of resurgence due to a loss of focus on eradication, or emerging resistance in the parasite, should not be understated.

### TRYPANOSOMES AND THE IMMUNE SYSTEM

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Unlike their close relatives, *Trypanosoma cruzi* and *Leishmania* spp., the African trypanosomes are strictly extracellular. As described above, they exist largely within the haemo-lymphatic and central nervous systems, while recent studies have shown that parasites can also be found in the skin and adipose tissue<sup>12,42</sup>. This means that they are exposed to the full immune activity of the host and must rely on extensive

and highly efficient mechanisms of immune escape. These include evading and manipulating both the cellular and humoral host responses in order to survive.

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### TRYPANOLYSIS

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Humans have trypanolytic factors which effectively lyse trypanosomes in the bloodstream<sup>43,44</sup>. The main such factor is apolipoprotein L1 (APO-L1). This forms a complex with various other factors, including a haptoglobin-related protein which is taken up by the trypanosome<sup>45</sup>. Although APO-L1 is the best-known trypanolytic factor there are also others which are less well understood<sup>46</sup>. These act as a front line of defence against trypanosomes, and people deficient in APO-L1 have been shown to be vulnerable to infection from trypanosome species such as *T. evansi* which do not normally infect humans<sup>47</sup>. The two human-infective forms of trypanosome, *T. b. gambiense* and *T. b. rhodesiense*, each has its own method of resistance to APO-L1. *T. b. rhodesiense* neutralises APO-L1 with the APO-L1 binding serum resistance associated (SRA) protein, a truncated Variant Surface Glycoprotein (VSG)<sup>48–50</sup>. In a similar fashion, the predominant sub-group of *T. b. gambiense* resists lysis through the binding of *T. b. gambiense*-specific glycoprotein encoded by the *TgsGP* gene<sup>51,52</sup> and also by reduced uptake of APO-L1 due to a single nucleotide polymorphism in the haptoglobin–haemoglobin receptor (HpHbR)<sup>53–55</sup>.

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### VSGS

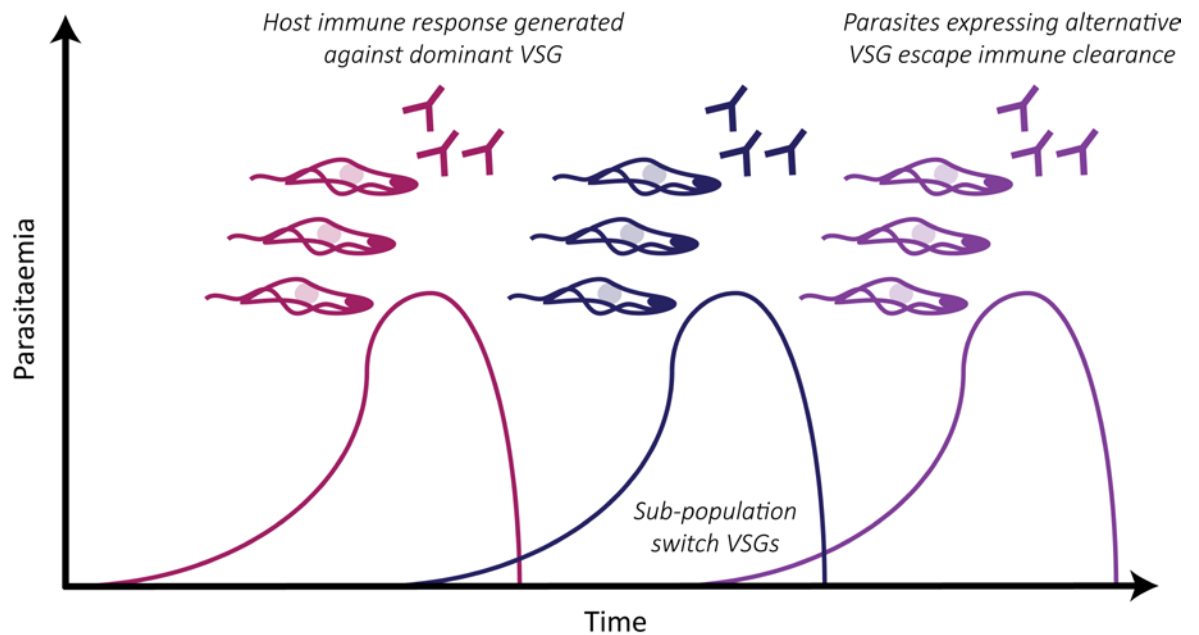
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VSGs are the parasite's primary defence against the host immune system. VSGs are 50-60 kDa homodimers with varying sequences and a conserved tertiary structure. The VSG coat is comprised of five million VSGs which are attached to the plasma

membrane with a glycosylphosphatidylinositol (GPI) anchor<sup>56</sup>. When the host raises an immune response to their highly immunogenic surface and clears the parasite, a small sub-population of trypanosomes will be expressing a different VSG on their surface. These parasites will be overlooked by the immune attack and will survive to continue to proliferate. This gives rise to the overlapping and repeating waves of immune clearance followed by rising parasitaemia that is characteristic of an African trypanosome infection (Figure 2). The parasite only expresses one of the 2000+ full or partial VSG genes at a time. This mono-allelic control is mediated through VSG exclusion protein 1 (VEX1)<sup>57</sup>, although the mechanisms are not yet fully understood. This is key in preventing the parasite from expressing multiple VSGs and maintaining its ability to 'escape' immune clearance.

This is not the only role that VSGs play in defending the parasite from the host immune response. VSGs are also used by *T. b. gambiense* to inhibit activation of the alternative pathway of complement by masking the sites that would trigger activation of the pathway and impair the ability of the host to lyse the trypanosome<sup>58,59</sup>. The rapid VSG recycling system that the trypanosome has in place also clears surface-bound complement factors and antibodies<sup>60</sup>. VSGs can be released into the bloodstream during peak parasitaemia, scavenging complement factors and potentially allowing the survival of a minority of parasites by preventing antibody-mediated complement activation<sup>61,62</sup>. This latter ability is thought to be the reason why GPI-PLC, the enzyme that cleaves the GPI-anchor and releases the VSG, has been found to be non-essential in cell-culture but has an important role in virulence<sup>63,64</sup>.

FIGURE 2: THE PATTERN OF PARASITAEMIA



Overlapping waves of parasitaemia typical of an African trypanosome infection. As the parasite multiplies within the host bloodstream, the immune response produces antibodies which target the VSG expressed on the cell surface. However, alternative VSGs will be expressed on the surface of some of the parasites within the population. As the antibody response does not target these alternative VSGs, the parasites expressing them survive the antibody-mediated clearance and multiply within the bloodstream, causing the repeating waves of parasitaemia within the host that are typical of an African trypanosome infection.

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## IMMUNOSUPPRESSION

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As well as this immune evasion by antigenic variation, trypanosome infections have been linked to general immunosuppression. The mechanisms by which the parasites induce this are not well understood<sup>65–67</sup>, but any immune suppression would obviously improve survival in the host. A couple of examples are better studied. In the initial stages of infection, one study showed that *Trypanosoma brucei* adenylate cyclase activity reduces the early innate defence of the host by inhibiting the synthesis of TNF- $\alpha$ <sup>68</sup>, similarly to other pathogens such as *Bacillus anthracis* (the causative agent of anthrax)<sup>69</sup>, and allowing the parasite to establish an infection in the host. A recent study has also shown that excretion of indolepyruvate by trypanosomes may modulate the host inflammatory response. Indolepyruvate decreases macrophage glycolysis and Hif-1 $\alpha$  production<sup>70</sup>.

One puzzling question is how the trypanosome guards against immune recognition of the large, non-VSG components on the cell surface. Components such as the moderately abundant invariant surface glycoproteins<sup>71–73</sup> or HpHbR mentioned above, are known to extend beyond the VSG surface and to be available for binding<sup>54,74</sup>. While it is known that *T. b. gambiense* HpHbR (as discussed above) is mutated in such a way as to reduce APO-L1 binding, it is not yet understood how it avoids antibody binding,<sup>54,75</sup> although its low expression in *T. b. gambiense* may help<sup>53</sup>. Indeed this last factor, combined with localisation predominantly in the flagellar pocket, is thought to provide the most likely answer to the question of how the trypanosome avoids immune recognition<sup>76–78</sup>, although the hydrodynamic flow of bound receptors that rapidly recycles VSGs may also contribute<sup>60</sup>.

Trypanosomes also have a range of methods to combat the adaptive immune response, such as impairing B-cell functionality and inducing early activation and polarisation of T-cells. These are beyond the scope of this study but are effectively reviewed by Stijlemans et al.<sup>79</sup>



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## TRYPANOSOMES IN CULTURE

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As can be seen, the African trypanosome has a complex relationship with its host. In addition to the immune interactions detailed above, it appears that trypanosomes alter the biochemical activity and behaviour of their hosts<sup>80–82</sup>. It has even been noted that *T. congolense*-infected animals are more frequently bitten by tsetse flies than non-infected animals. Some have conjectured that this is due to trypanosome catabolites which are secreted in the urine of infected hosts to make the host more attractive to the tsetse fly<sup>83,84</sup>. *T. brucei* is also known to stimulate the production of interferon  $\gamma$  by host lymphocytes<sup>85</sup>. This is then capable of stimulating *T. brucei* proliferation<sup>86</sup>.

During a trypanosome infection, the host's physiology can be distinctly altered, so questions have been raised as to whether a culture medium can accurately reflect the complexities of the *in vivo* infection. It is known that several bacterial species express a different gene set in their mammalian hosts than in the culture flask<sup>87</sup> and, for example, rates of adaptation to antibiotic resistance in *Salmonella* spp. and even the preferred mutations that confer resistance can vary from host to flask<sup>88</sup>. Could similar complexities exist with trypanosomiasis?

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## THE DEVELOPMENT OF *IN VITRO* CULTURE

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Although earlier attempts are recorded<sup>89,90</sup>, 1967 is the first time that short-term culture in the presence of feeder cells was observed for *T. brucei*<sup>91</sup>. It took a further ten years before continuous propagation was observed<sup>92</sup>, also in the presence of feeder cells. However, this method only worked with the Lister 427 strain of *T. brucei*.

The introduction of Minimal Essential Medium of Eagle (MEM) with supplemental amino acids, glutamine, embryonic fibroblast-like cells from the north American vole, and heat-inactivated human serum allowed the culture of other strains of *T. brucei*<sup>93,94</sup>. The recognition by Duszenko and colleagues in the Cross laboratory that supplementation with cysteine could eliminate the requirement for a feeder cell layer<sup>95</sup> simplified culture techniques substantially. Further experimentation showed that the addition of reducing agents helped to stop this addition of cysteine from being toxic to the cells<sup>96</sup>. Cysteine was later shown to be an essential amino acid for trypanosomes<sup>97</sup>.

In 1989, Hirumi and Hirumi published their paper detailing the HMI-11 medium which is still used in laboratories today<sup>98</sup>. Based on Iscove's modification of Dulbecco's MEM (IMDM) and adding a copper chelator to prevent the oxidation of cysteine, HMI-11 is a rich medium with a large overabundance of key nutrients.

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#### THE LIMITATIONS OF *IN VITRO* CULTURE

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This medium is foundational to most trypanosome experiments, including the screens used to find new drugs for trypanosomiasis. However, the medium is much richer than the actual situation in an infection *in vivo* (Table 1). This leads to several problems. For example, the high concentration of common metabolites means that it is difficult to observe metabolic alterations because of the large background pool. Drug-induced perturbations are also often missed due to the masking effect of compensatory mechanisms. Immune attack by the host is not present in culture, and it is reasonable to assume that several metabolic stresses are also missing from the standard culture system. There may well also be certain activities in rich culture media that are not

relevant to the medical situation. The complex interactions described above where the trypanosome manipulates its host are also missing from the lab-based culture media.

TABLE 1: KEY COMPONENTS OF HMI-11 COMPARED TO HUMAN BLOOD

<i>Component</i>	<i>HMI recipe</i>	<i>Human Blood</i>	<i>Excess</i>
<i>Glucose</i>	25,000	4,817	5x
<i>L-Cysteine</i>	1,504	99	15x
<i>L-Methionine</i>	201	29	7x
<i>Hypoxanthine</i>	1000	8	125x
<i>Folic Acid</i>	9	0.03	300x
<i>Thymidine</i>	161	0.2	805x
<i>Myo-inositol</i>	40	24	1.6x

Table showing levels of key components of HMI-11 compared to average levels in human blood. Adapted from Creek et al.<sup>99</sup>, data taken from the Human Metabolome Database<sup>100–102</sup>. All data in  $\mu\text{M}$  concentrations

It is highly likely that many of the previous high-throughput screening approaches using the standard HMI-11 media may have underestimated the potency of many potential drugs, especially where those are connected to metabolic processes or immune evasion<sup>99</sup>. For example, this has been observed previously in the study of dihydrofolate reductase (DHFR)-thymidylate synthase inhibitors where it was shown that these inhibitors only show significant trypanocidal activity where folic acid is depleted<sup>103</sup>.

Metabolomics has proved to be a useful tool for the rational optimisation of cell culture medium. Using this approach has led to media called Creek's Minimal Media (CMM) which allows the screening of trypanocidal activity by established methods but in a medium that more closely represents metabolite concentrations in human blood<sup>99</sup>.

## THIS PROJECT

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### RNAI SCREENS

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Genome-wide RNAi library screens have proved highly useful for probing trypanosome biology: in particular for elucidating drug resistance mechanisms<sup>104,105</sup>. An unpublished screen carried out in David Horn's laboratory found 45 genes which were essential only *in vivo*. These hits form the basis for this project. The screen used an RNAi plasmid library comprised of randomly fragmented genomic DNA<sup>106</sup> to create a library of inducible RNAi in bloodstream *T. brucei*. This was then used to determine essential genes as previously described for the published *in vitro* screen<sup>107</sup>. However, the trypanosomes were inoculated into rats rather than grown in culture flasks (Figure 3A).

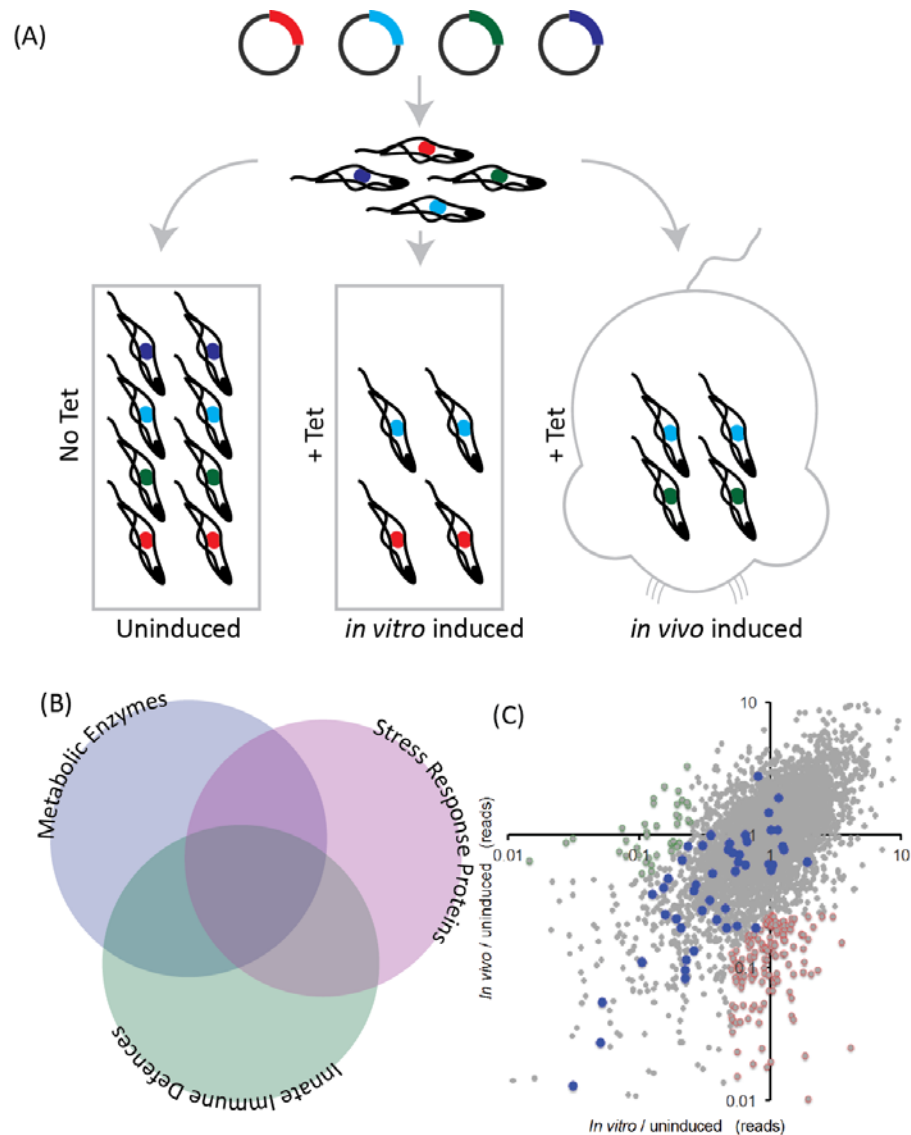
A control set of genes whose dispensability or essentiality was known to be the same both *in vitro* and *in vivo* was used to establish cut-off points to delineate the hits which were of interest. This gave a list of forty-five hits (Table 5; Appendix 1) which were essential *in vivo* while being dispensable in standard culture conditions. The screen has not been carried out *in vivo* before and therefore there is still extensive verification to be done in order to validate the data set.

We expected to see a number of genes from categories such as innate immune defences, stress and metabolism as well as some which show overlapping functions within these categories (Figure 3B). We also expected to find some of the few proteins which have been previously shown in the literature to have an *in vivo*-specific function.

We discovered genes which played a role in phosphatidylinositol signalling and in control of allelic exclusion, the method by which the parasite maintains the

effectiveness of the VSG coat in avoiding immune clearance. These genes were inositol-1(or 4)-monophosphatase 2 (IMP)<sup>108</sup>, glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC)<sup>64</sup> and a histone-lysine N-methyltransferase (DOT1B)<sup>109</sup>. This was encouraging as it met our expectations.

FIGURE 3: RNAi SCREEN DIAGRAM



(A) Diagram of the RNAi screen showing RNAi library induced in both *in vivo* and *in vitro* situations. The **dark blue** and **light blue** populations represent genes essential or dispensable respectively in both the *in vitro* and *in vivo* scenarios. **Green** and **red** populations represent genes which are only essential in the *in vitro* and *in vivo* conditions respectively. The latter population is the one of interest for this project. (B) Expected categories of proteins which show a specifically *in vivo* essential phenotype. (C) Graphical representation of the results from the RNAi screen. The **blue** population shows control genes, many of which are associated with a fitness cost both *in vitro* and *in vivo* (intraflagellar transport, proteasome, nuclear pore, MCM2-7 and coatomer components). **Green** and **red** populations represent genes which are only essential in the *in vitro*/*in vivo* conditions, respectively.

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## AIMS AND OBJECTIVES

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My primary aim was to improve understanding of host:*T. brucei* interactions at the molecular level. In order to do this I aimed:

- To analyse hits from the *in vivo* RNAi screen.
- To develop a culture method more resembling the *in vivo* situation.
- To use the new culture method to validate a small number of hits from the screen.



## EXPERIMENTAL PROCEDURES

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### BIOINFORMATICS AND HIT PRIORITISATION

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In order to understand the function of products which were encoded by the genes on our hit list, the gene IDs were used to query the publicly available datasets on TriTrypDB<sup>110</sup>. Existing data on the function and characteristics of the gene products, such as conservation among trypanosomes, the number of predicted transmembrane domains and potential orthologues in other species, were compiled. Data from transcriptomic and proteomic studies analysing differential protein<sup>111</sup> and RNA expression<sup>112</sup> between *T. brucei* developmental stages were also considered, as it was thought likely that genes up-regulated in bloodstream forms were more likely to be genuinely involved in host:parasite interactions.

BLAST<sup>113</sup> was used to assign putative functions based on sequence similarity and to assess the conservation of each gene by searching the 'all organisms' database by gene and protein sequences and recording whether they were conserved only among the trypanosomes, among other parasites, or more broadly. Each hit was then searched against the NCBI Conserved Domain Database (CDD)<sup>114–116</sup> to find any known domains which could inform function and assign potential pathways.

## STRAIN GENERATION

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Genomic DNA was isolated from bloodstream *T. brucei* using a DNeasy kit (Qiagen) according to the manufacturer's instructions. From this genomic template, fragments of 400–600 bp targeting the genes of interest were amplified by high-fidelity Phusion polymerase (NEB) according to the manufacturer's instructions (Figure 4A). These fragments were ligated overnight at 4°C using T4 ligase (NEB) according to manufacturer's instructions in sense and antisense configurations (Figure 4E) to digested pRPa<sup>iSL</sup> (117) to generate inducible stem-loop RNAi constructs (Figure 4C, D). For tagging at the native loci, C-terminal fragments were amplified (Figure 5A) and cloned into pNAT<sup>xTAG</sup> (117) (Figure 5C) and verified by western blot. PCR primers were designed using RNAit<sup>118</sup> (Table 2). All oligonucleotides were synthesised by ThermoFisher Scientific.

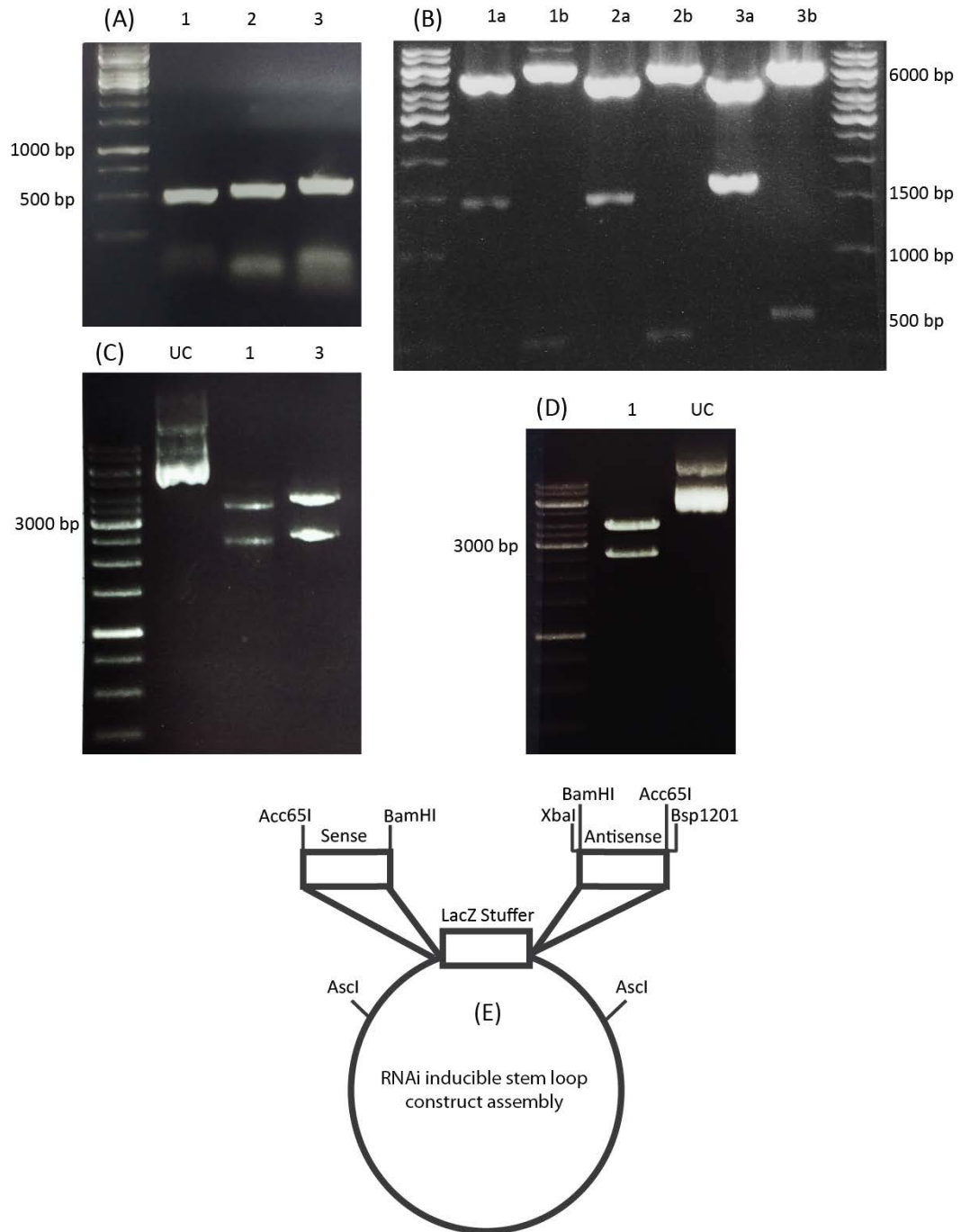
Bloodstream-form *T. brucei* 2T1 cells<sup>117</sup> were transfected using a Nucleofector™ 2b (Lonza) with Cytomix. Clonal populations were generated by limiting dilution and selected with hygromycin (2.5 µg/mL) for RNAi constructs successfully integrated at the tagged locus and with blasticidin (10 µg/mL) for native tagging constructs (Table 3) according to the standard procedures<sup>117,119</sup>.

TABLE 2: PRIMER SEQUENCES

Primer	Sequence	Notes
<i>F_RNAi_5.2690</i>	GATCGGGCCC <u>GGTACCT</u> GCGTCTCTATAGCCCTCGT	<b>Bsp1201</b> <u>Acc651</u> 5' sequence
<i>R_RNAi_5.2690</i>	GATCTCTAGAGGATCCTAGCTGCAACTCCCACTCCT	<b>Xba1</b> <u>BamH1</u> 3' sequence
<i>F_RNAi_10.4260</i>	GATCGGGCCC <u>GGTACCT</u> TCACTGATCAGTCGCAAGG	<b>Bsp1201</b> <u>Acc651</u> 5' sequence
<i>R_RNAi_10.4260</i>	GATCTCTAGAGGATCCTGCAGCCTTTTATTGTGCAG	<b>Xba1</b> <u>BamH1</u> 3' sequence
<i>F_RNAi_8.2610</i>	GATCGGGCCC <u>GGTACCC</u> GGCGGTGCGATAAATGAGG	<b>Bsp1201</b> <u>Acc651</u> 5' sequence
<i>R_RNAi_8.2610</i>	GATCTCTAGAGGATCCCGTTAATATGCCCTTCACT	<b>Xba1</b> <u>BamH1</u> 3' sequence
<i>F_8.2610.12MYC</i>	GATCGGCGCGCCATTGATTCCGACAACTACG	Native tagging, <b>Ascl</b>
<i>R_8.2610.12MYC</i>	GATCTCTAGAGTGCACAGTAAAGTGCCGCT	Native tagging, <b>Xba1</b>
<i>F_5.2690.12MYC</i>	GATCAAGCTTATGCCAATAAATAACTGTGA	Native tagging, <b>HindIII</b>
<i>R_5.2690.12MYC</i>	GATCTCTAGAGCATCCTAACACCGATTTTT	Native tagging, <b>Xba1</b>

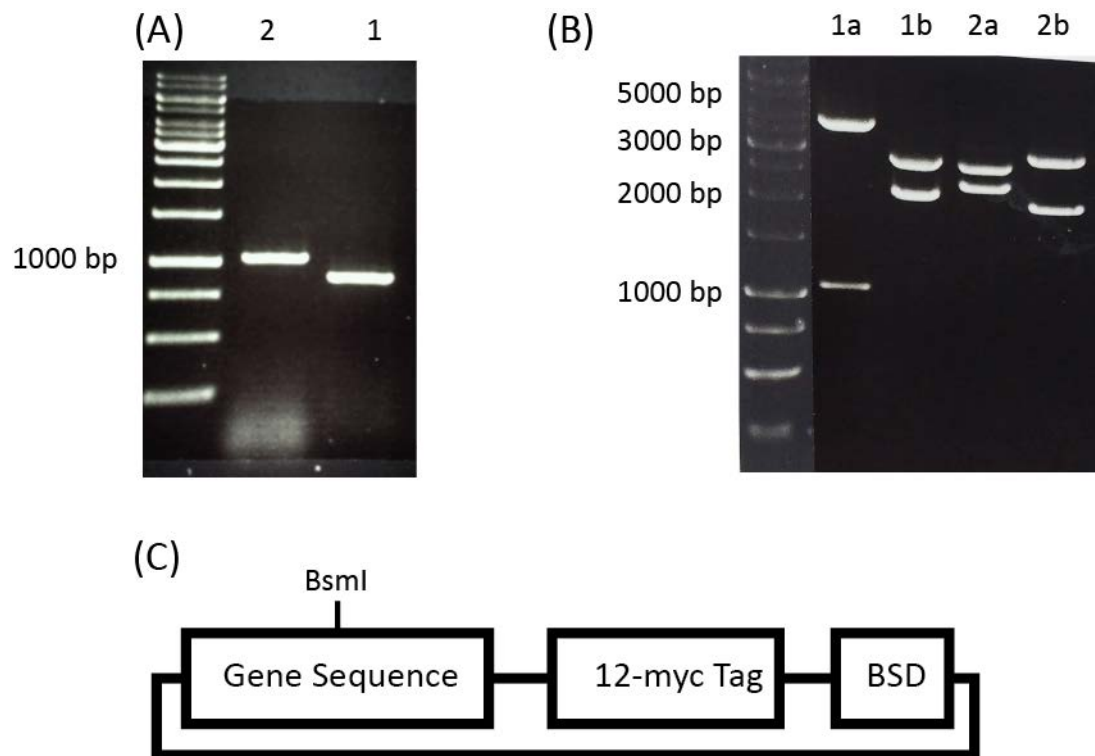
Primer Sequences used in construct assembly.

FIGURE 4: RNAi CONSTRUCT ASSEMBLY



Assembly of RNAi constructs. 1: IMP. 2: 5-MetE. 3: Hyp-Con. (A) PCR products. All show expected sizes (approx. 1 – 500 bp, 2 – 550 bp, 3 – 600 bp). (B) Test digests. a: Acc65I, releasing stuffer and both inserts. b: Bsp1201 and XbaI, releasing second stage insert. All show expected sizes (approx. 1a – 1400, 5000; 1b – 400, 6000; 2a – 1500, 5000; 2b – 500, 6000; 3a – 1700, 5000; 3b – 600, 6000) (C) Ascl digest to release construct for integration into parasite genome. From left to right: uncut control, IMP and Hyp-Con. (D) Ascl digest. From left to right: MetE, uncut control. (E) Diagram showing construct assembly.

FIGURE 5: TAGGING CONSTRUCT ASSEMBLY



(A) PCR for native tagging showing expected sizes. 1: MetE, 1010 bp. 2: IMP, 850 bp. (B) Test digests for the final constructs. 1a: IMP, MluI digest; 1, 4.2 kbp. 1b: IMP, AclI digest; 2.2, 2.9 kbp. 2a: 5-MetE, Sall digest; 2.4, 2.9 kbp. 2b: 5-MetE, SspI digest; 2.1, 3.2 kbp. (C) Diagram showing construct design. BsmI site in both constructs is used to linearise the plasmid for integration into the genome at the target locus. BSD: Blasticidin resistance cassette.

## TRYPANOSOME CULTURE

Initial tests were with trypanosomes grown in calf serum (Sigma) supplemented with bathocuproinedisulfonic acid (30  $\mu$ M) and beta-mercaptoethanol (10  $\mu$ l/L). Otherwise, trypanosomes were grown in HMI-11<sup>98</sup>, or calf serum (Sigma) supplemented with bathocuproinedisulfonic acid (30  $\mu$ M) and thioglycerol (10  $\mu$ l/L). Calf serum was filtered before supplementation where noted. Folate, thymidine and methionine-free media were prepared as per the standard HMI-11 recipe, with the omission of folic acid, thymidine, and methionine as appropriate. Cumulative growth curves were carried out using cultures seeded at  $10^4$  or  $10^5$  cells/mL as appropriate, counted on a haemocytometer (Neubauer) every 48 h or 24 h respectively, and diluted as necessary. Induction of RNAi constructs was by 1  $\mu$ g/ml tetracycline. Transgenic parasites were selected for and maintained using the appropriate drug concentrations detailed in Table 3. Standard incubator conditions of 5% CO<sub>2</sub> and 37°C were used for all cell culture.

TABLE 3: DRUG CONCENTRATIONS

	Construct	Selection	Maintenance
<i>Hygromycin</i>	RNAi	2.5	1
<i>Phleomycin</i>	All	1	0.2
<i>Blasticidin</i>	Tag	5	1
<i>Puromycin</i>	2T1	2	1

Drug concentrations used for selection and maintenance of bloodstream *T. brucei*. All concentrations in  $\mu$ g/ml.

## TAG AND PROTEIN ANALYSIS

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### WESTERN BLOTTING

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$1 \times 10^7$  cells were pelleted by centrifugation at 1,000 g for 10 minutes. HMI-11 was removed and the pellets were washed twice with 1x PBS to remove trace elements of media. Pelleted samples were then resuspended in SDS sample buffer at a concentration of 100,000 cells/ $\mu$ l and proteins denatured by boiling for 5 minutes.

Protein samples were loaded equally on two 8% polyacrylamide gels and run at 150 V for approximately 90 minutes. One gel from each experiment was then fixed with isopropanol for 15 minutes, stained with coomassie blue (1-3 h) and destained overnight for a loading control. The second gel was soaked for 15 minutes in transfer buffer (48 mM Tris, 39 mM glycine, 20% Methanol, 0.0375% SDS) and transferred to nitrocellulose at 20 A for 20 min.

Nitrocellulose membranes were blocked in 5% Marvel in TBS-T (0.05% Tween) overnight at 4°C. 12-myc tags were detected using 1:5000 dilutions of mouse anti-myc antibody (Source Bioscience) and 1:2000 dilutions of goat anti-mouse antibody (BioRad).

### IMMUNOFLUORESCENCE

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500  $\mu$ l of log-phase culture was added to 500  $\mu$ l 2% paraformaldehyde and incubated at 4°C for 1 h before being pelleted at 6000 rpm. Pellets were washed three times in ice-cold PBS before being resuspended in 1 ml 1% Bovine Serum Albumin and spotted on slides.

After drying overnight, cells were washed in PBS and permeabilised using 0.5% Triton X-100 in PBS. The cells were then blocked in 50% FBS.

12-myc tags were detected using mouse anti-myc antibody (Source Biosciences) diluted in 3% FBS and FITC labelled anti-mouse antibody (Thermo) diluted 1:100 in 3% FBS. VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories) was used to mount the cells and stain the DNA.



## RESULTS

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### RNAI SCREEN ANALYSIS

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We found seven genes for which homology and conserved domain searches indicate a role in metabolism. Three of these appear to have a role in Folate/Methionine metabolism. Five of the hits have a role in either cell cycle checkpoints or the stress response. Only three genes are thought to be linked to the immune system. However, the innate immune defence of the parasite has not been extensively studied. Given that the functions of eighteen out of the forty-five genes selected by this screen are completely unknown, there could be many new innate immune defence factors in this large section of the dataset. However, we did find two genes which are known to have a role in immune defence: GPI-PLC<sup>64</sup> and DOT1B<sup>109</sup>. GPI-PLC is involved in releasing VSG into the bloodstream, while DOT1B is crucial in the modulation of antigenic variation and mono-allelic expression. The centrality of both of these to the parasites' immune defence mechanisms is discussed further below. Lists of hits from each of the above categories and further information are shown in Appendix 1.

A search of the dataset using TriTrypDB revealed 49% of the dataset to be annotated as being either hypothetical proteins or proteins containing a domain of unknown function. However, this was not a statistically significant difference compared to 35% when the same search was carried out against the whole genome ( $P = 0.0541$ ).

In order to select hits from the *in vivo* RNAi screen described above for a pilot study, particular features were looked for such as the presence of a reasonable hypothesis as to why these genes proved dispensable *in vitro* and essential *in vivo*; conservation among the kinetoplastids; the presence of signal peptides or transmembrane domains (Table 5; Appendix 1: RNAi Screen Hits), indicative of the gene product being targeted for secretion or being external to the surface of the parasite; and lastly, the quality of the coverage in the RNAi screen (Figure 6).

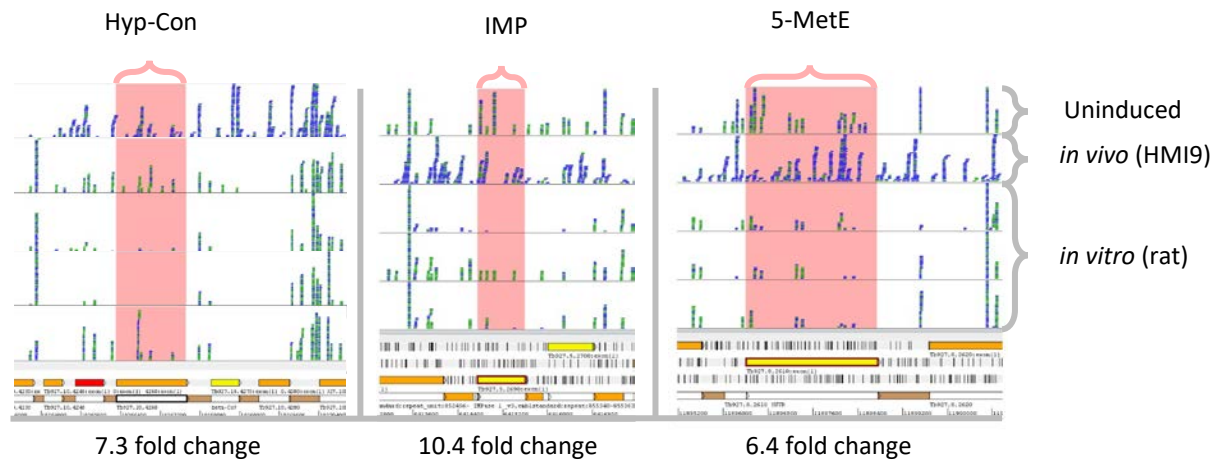
Examining this led us to prioritise three hits for initial validation. These hits were: Tb927.5.2690, a putative inositol monophosphatase (IMP) selected for its link to the parasite immune defence via *myo*-inositol synthesis; Tb927.8.2610, a putative methyltransferase (5-MetE), selected for its links to the crucial *in vivo* process of folate metabolism; and Tb927.10.4260 (Hyp-Con), a hypothetical protein selected for its large number of transmembrane domains and possible role in lipid transport. This latter possibility was highlighted by sequence similarity which indicated that the protein was similar to a known protein family containing a lipocalin transmembrane receptor.

TABLE 4: PILOT GENES

Name	Gene ID	# TM	SignalP	# Orthologues	mRNA <sup>112</sup>	Proteome <sup>111</sup>
IMP	5.2690	0	null	29	1.155	2.91
5-MetE	8.2610	0	null	24	1.698	No data
Hyp-Con	10.4260	10	0.9	23	No data	8.91

Table showing known characteristics of the three genes chosen for pilot study. #TM describes the predicted number of transmembrane domains from the sequence. SignalP describes the probability of the gene containing a signal peptide for secretion/expression on the surface from sequence similarity to known signal peptides. #Orthologues shows the number of known orthologues to the gene across sequenced genomes. mRNA describes fold change in mRNA abundance (bloodstream/procyclic). Proteome describes fold change in protein abundance (bloodstream/procyclic)

FIGURE 6: SEQUENCE READ MAPPING



Sequence read mapping for the three hits pursued as part of this study. High-throughput RIT-seq carried out for all five populations (uninduced, *in vitro* induced and 3x *in vivo* induced) and the data mapped by Axel Martinelli at Sanger according to previously described protocols<sup>120</sup>. Numbers for fold change refer to the difference between the average of the three *in vivo* induced populations and the *in vivo* induced sample.

## GROWTH IN SERUM TO MIMIC THE *IN VIVO* ENVIRONMENT

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Previous work by Nicola Baker in this lab indicated linear growth of trypanosomes was achievable in FBS (Sigma) supplemented with a copper chelator, bathocuproinedisulfonic acid (30  $\mu$ M); and a reducing agent,  $\beta$ -mercaptoethanol (14  $\mu$ l/L). However, this was not readily reproducible.

I decided to test this and also whether the substitution of thioglycerol for the reducing agent in place of  $\beta$ -ME would be more reproducible. Following several attempts, with different batches of serum, I found that bathocuproinedisulfonic acid and thioglycerol (14  $\mu$ l/L) provided stable and linear growth with the limitation that the parasites could not be seeded below  $10^5$  / ml and therefore required daily splitting. Parasites seeded at lower densities did not grow (data not shown). Serum with  $\beta$ -mercaptoethanol failed to show linear growth (Figure 7). I also tested Creek's Minimal Media<sup>99</sup>. The test proved unsuccessful (Figure 7A), for reasons which remain unknown.

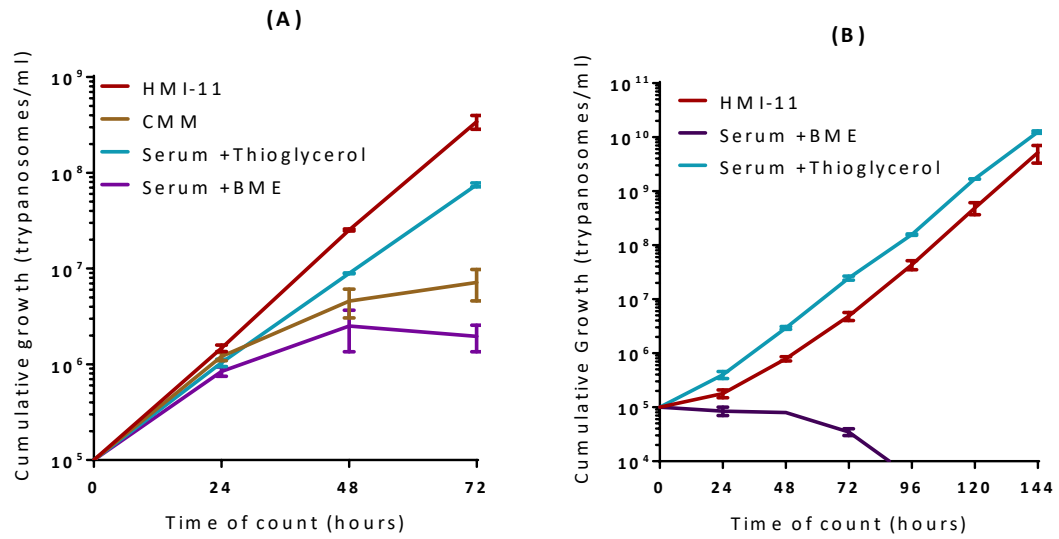
More recent experiments indicated that the presence of cysteine (1.5 mM) in the culture medium provided faster and more stable growth. However, this was not reflected in the repeat experiments (data not shown). This difference may have been due to the different environment provided by using a plate instead of a flask to grow the cells in the former experiment. Further experiments are required to verify the impact of cysteine on the serum system.

In order to confirm these results over a longer period, growth curves were set up to compare growth in HMI-11 with sterile filtered (in order to deal with a contamination issue) serum supplemented in three different ways. The cells were grown under

standard conditions and were counted and split to  $1 \times 10^5$  daily. The results (Figure 7B) showed that serum supplemented with bathocuproinedisulfonic acid and thioglycerol (14  $\mu\text{l/L}$ ) yields robust growth comparable to that in standard rich media conditions and which can be maintained over a six-day period. This simple serum medium was termed SS1 for the duration of the study. As sterile filtered serum appeared to provide superior growth (Figure 7B) compared to the initial tests with non-sterile filtered serum (Figure 7A), it was used for the remainder of the study. The reason for this effect is unknown; but it may be due to the removal of low-grade bacterial contaminants which were previously unobserved. Alternatively, it could simply be due to variation in serum batches. In order to control for this in future, the remainder of the batch was reserved for all future experiments using SS1.

Other evidence from a course run by members of this lab at the Marine Biology Laboratory in Woods Hole, MA suggests that it is possible to grow trypanosomes in serum supplemented with beta-mercaptoethanol at a similar level to that shown in this report with thioglycerol-supplemented serum. We should, therefore, like to repeat this to address the question of whether these conflicting reports are due to variation between serum sources.

FIGURE 7: ALTERNATIVE MEDIA DEVELOPMENT

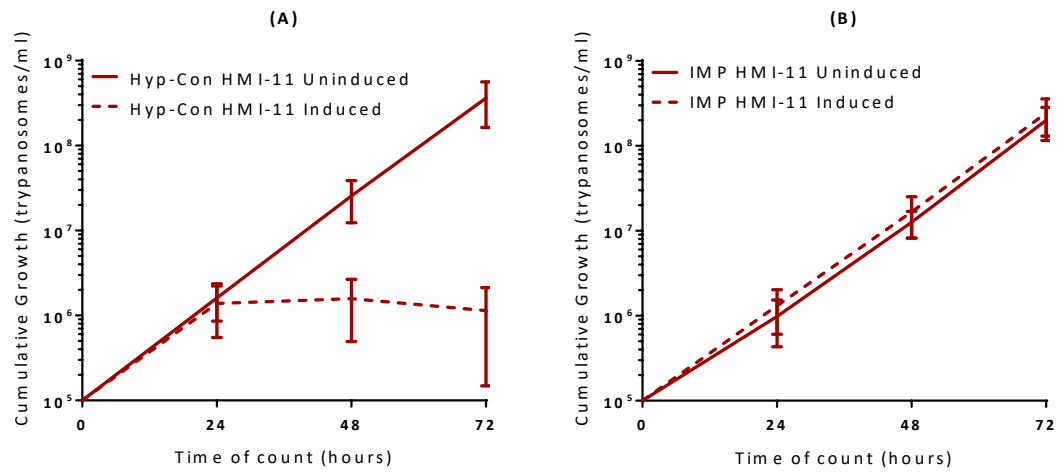


Graphs showing initial assessment of potential media for studying the hits from the *in vivo* screen. Two technical replicates using 2T1 wild-type cells were used for each condition in both experiments. Error bars show standard deviation from the mean of two independently generated biological clones. (A) Initial media assessment showing parasite growth in HMI-11, CMM and serum supplemented with  $\beta$ -ME or thioglycerol. (B) The growth of wild-type trypanosomes in HMI-11 and serum supplemented with  $\beta$ -ME or thioglycerol. In both experiments Serum +Thioglycerol: serum supplemented with bathocuproinedisulfonic acid (30  $\mu$ M) and thioglycerol (14  $\mu$ L/L). Serum +BME: Replacement of thioglycerol with beta-mercaptoethanol (14  $\mu$ L/L).

In order to discover whether the genes were required *in vitro*, RNAi strains were assembled and induced over a three-day period in HMI-11. This experiment showed that the hypothetical conserved protein encoded by Tb927.10.4260 was not a valid hit from the screen, as it showed a clear *in vitro* growth defect (Figure 8). Due to this, it was not of interest for this study and no further experiments with this gene are planned.

The induction of RNAi targeting the putative inositol monophosphatase (IMP) showed no such growth defect, and the parasites grew the same both when induced and uninduced. At this stage, 5-MetE was not tested as the construct assembly was incomplete. Data for this gene is shown below (Figure 10).

FIGURE 8: CUMULATIVE GROWTH OF RNAI STRAINS IN HMI-11



Cumulative growth over 72 hours of two initial RNAi strains in HMI-11. Error bars show standard deviation from the mean of two independently-generated biological clones.

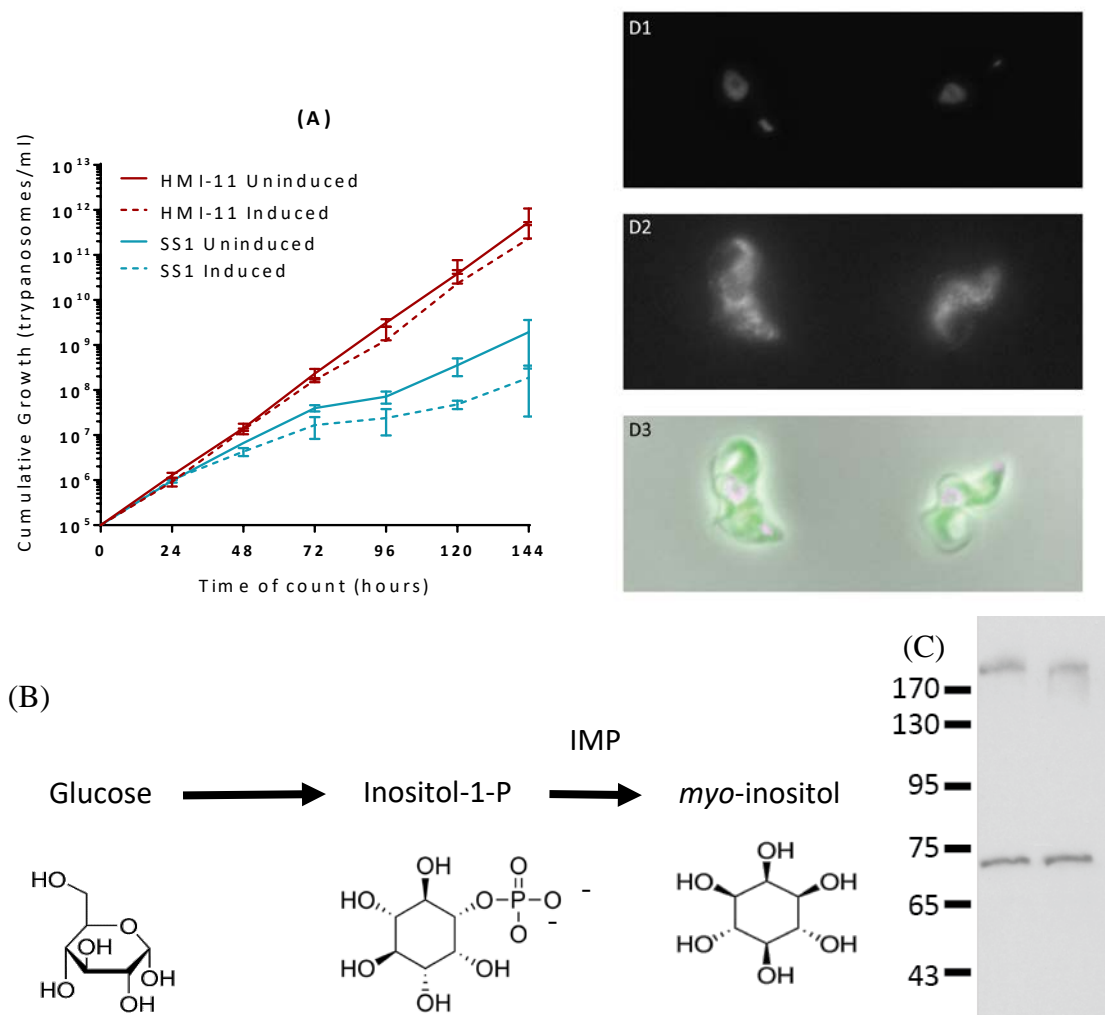


In order to test whether the phenotype was different in SS1 from the standard media conditions, two independent biological clones containing the inducible stem-loop construct targeting the putative inositol monophosphatase (IMP) gene were induced by the addition of tetracycline at 1 µg/ml in HMI-11 and SS1. The cells were counted every day and split to a density of  $10^5$  cells per ml.

There was higher than expected variability between the two clones; but despite this, I observed a difference between the phenotypes in HMI-11 and serum on induction (Figure 9A). Due to the variation between the clones, I repeated this experiment. However, a loss of tetracycline control in one of the RNAi cell lines meant that the stem-loop RNAi could not be induced. However, this hit has since been validated independently in work by Cestari et al., who showed that IMP is non-essential in media, but essential in mouse models<sup>108</sup>. So it was not considered necessary to generate new RNAi cell lines and repeat this experiment. The most obvious hypothesis generated from these results is that synthesis of inositol from glucose via IMP (Figure 9B) is not necessary for parasites in HMI-11 due to its overabundance of inositol compared to human serum (Table 1).

The IMP gene was successfully tagged and verified by Western Blot (Figure 9C), although it migrated slower than expected (expected size 50 kDa). Immunofluorescence analysis appears to show localisation throughout the cytosol (Figure 9D). This localisation is also seen in the procyclic form via the TrypTag initiative<sup>121–123</sup> (Appendix 1: RNAi Screen Hits; Figure 1, Table 6). This localisation is concurrent with a protein involved in inositol metabolism.

FIGURE 9: IMP RNAI AND PROPOSED PATHWAY



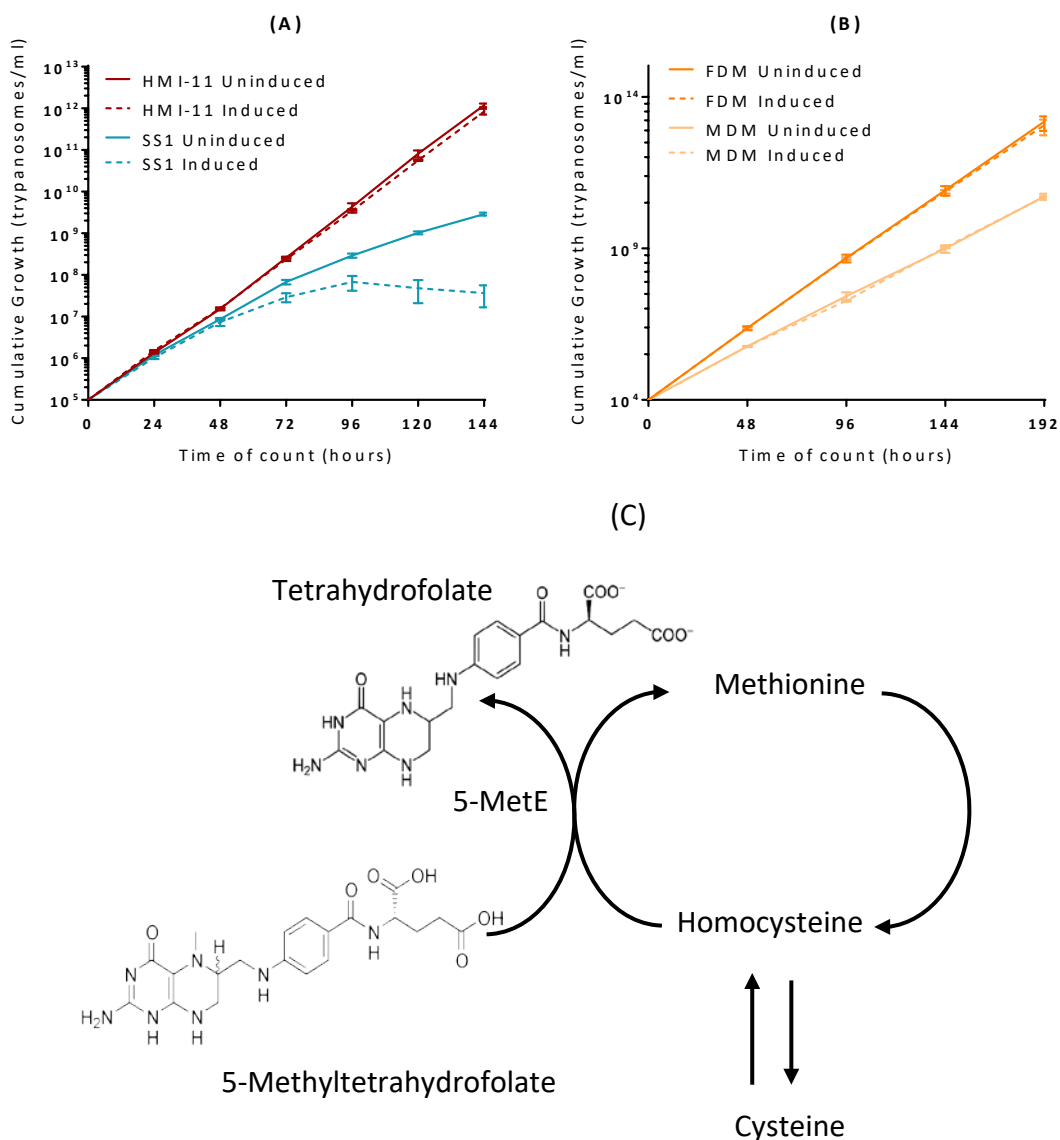
(A) Cumulative growth over six days of two independent RNAi strains targeting Tb927.5.2690. Error bars show standard deviation from the mean of two independently-generated biological clones. (B) A simple diagram showing proposed pathway which the RNAi interrupts. (C) Western Blot showing successful tagging of IMP in two separate clones. (D) IFA showing localisation of IMP. D1: Dapi, D2: 12-myc tagged IMP, D3: Combined.

The second gene tested for a serum growth defect was Tb927.8.2610, which encodes a putative 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase (5-MetE). There was little variability between the clones and a clear growth defect on tetracycline induction at 1 µg/ml which is not seen in HMI-11 (Figure 10A).

5-MetE is required to convert 5-methyltetrahydrofolate into a form of folate which the trypanosome is able to utilise by removing the methyl group, which is then transferred to homocysteine to form methionine (Figure 10C). Either or both of these roles could be key in the gene's essentiality *in vivo* because both methionine and folic acid are overabundant in HMI-11 compared to human blood (Table 1). Methionine levels are seven times higher than in blood and folic acid is three hundred times higher. Thymidine, which provides the parasite with a pathway that can bypass folate metabolism, is 805x times overabundant. Any of these nutrients could be key to the dispensability of this otherwise essential gene.

In order to test these hypotheses, folate-free medium was prepared according to the standard recipe for HMI-11 with the omission of folic acid and thymidine. The growth curve was continued for eight days and the cells split to  $10^4$  / ml every 48 h. When this provided no phenotype (Figure 10B), it was repeated under the same conditions using methionine-free media prepared according to the standard HMI-11 recipe with the omission of methionine, folic acid and thymidine. Again, no phenotype was observed (Figure 10B). Given the possibility of other compensatory mechanisms, another experiment was designed to probe the system. Tagging for this gene was not successful.

FIGURE 10: 5-METE RNAI AND PROPOSED PATHWAY



Cumulative growth curves for RNAi strains targeting 5-MetE. Error bars show standard deviation from the mean of two independently-generated biological clones. (A) Cumulative growth over six days of two independent RNAi strains targeting Tb927.8.2610. (B) Cumulative growth following RNAi knockdown of 5-MetE in media lacking folate and thymidine (FDM) as well as media lacking folate, thymidine and methionine (MDM). (C) Simplified Folate/Methionine metabolism diagram showing the proposed action of 5-MetE which Tb927.8.2610 RNAi disrupts.

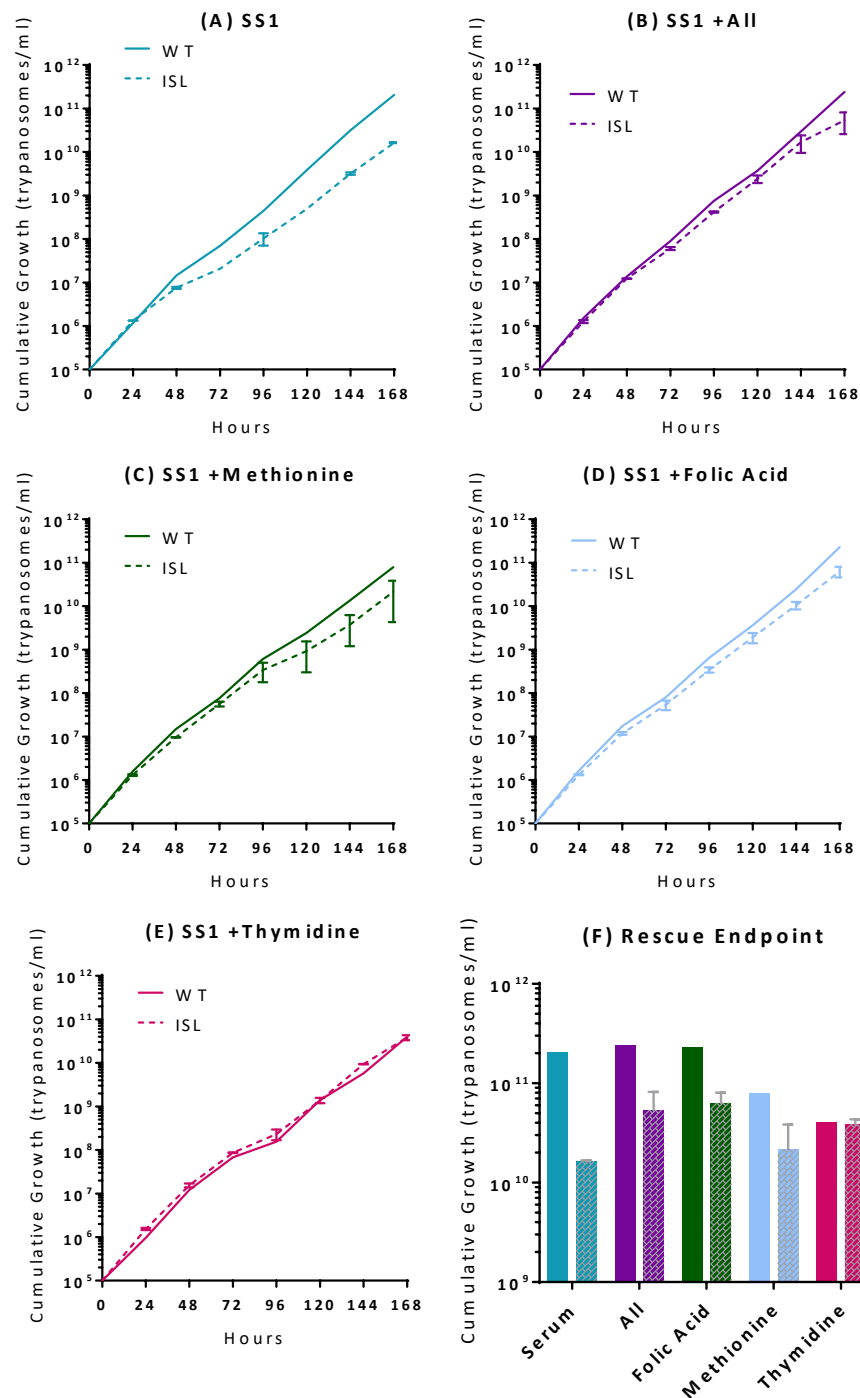
## 5-METE DEPLETION MAY BE RESCUED BY METABOLITE SUPPLEMENTATION

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The four supplements judged most likely to rescue the RNAi phenotype (Folic Acid, Glucose, Methionine, and Thymidine) were picked and growth curves carried out in duplicate for each singly and for all four combined. The supplements were added to the basic serum system containing thioglycerol and bathocuproinedisulfonic acid. Concentrations for each supplement were chosen to match the levels used in making HMI-11, in which I previously showed that there was no RNAi phenotype (Figure 10). As our hypothesis is that the enzyme is required to convert the folic acid found in serum and human blood to a useable form, forming methionine as a useful by-product of this process, we should expect an increase in viability under RNAi induction in SS1 when grown with these supplements.

Although the results trended in the expected direction (Figure 11), the RNAi cell line is not as responsive as previously observed (Figure 10A), and the results are not statistically significant. This is potentially due to loss of tetracycline control, something which has been observed in several laboratories with older RNAi strains.

FIGURE 11: RESCUE OF RNAI PHENOTYPE IN SS1



Rescue of RNAi phenotype using targeted supplementation of serum culture system. All growth curves generated at the same time under the same conditions. Solid lines show wild type data, dotted lines show induced RNAi targeting Tb927.8.2610. Error bars show standard deviation from the mean of two independently-generated biological clones. (A-E) Trypanosome growth in SS1 supplemented with 0.009 mM folic acid (D), 0.2 mM methionine (C), 0.16 mM thymidine (E) or all three in combination with glucose (B). (F) Total growth over the course of the experiment for each of the populations. Solid bars show uninduced, shaded bars show induced data.

## DISCUSSION

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### TRYPANOSOMES AND THE HOST

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African trypanosomes are extremely important pathogens due to the devastating societal and economic impact that they have in sub-Saharan Africa. If untreated, trypanosomiasis is fatal and the current treatment options are highly inadequate. In consequence, the development of new drugs and improved diagnostics is of vital importance.

Our goal was to improve our understanding of the interplay between the host and the pathogen at the molecular level. We suspected that due to the differences between the culture media and the *in vivo* situation, many areas of potential drug discovery could have been missed in previous screening approaches. Therefore, we hoped that by increasing our understanding of *T. brucei*:host interactions, novel avenues of drug discovery could be uncovered.

Past uses of high-throughput phenotyping approaches such as RNAi target sequencing (RIT-seq) to open up new aspects of trypanosome biology for further study have proved extremely effective. We therefore chose to use a genome-wide RNAi fragment library to probe for differences between the *in vivo* and *in vitro* essential gene sets. The unbiased approach given by RIT-seq is highly suited for investigation of an as yet unexplored area such as this.

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### TRYPANOSOMES AND THE IMMUNE SYSTEM

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The African trypanosomes are strictly extracellular organisms. This has forced them to develop a complex array of anti-immune defences in order to prevent the host

immune system from lysing them via trypanolytic factors<sup>43,44</sup>, antibody-mediated lysis<sup>124,56,57</sup> and complement<sup>58,59</sup>. The trypanosome also has a range of processes which have been linked to general immunosuppression<sup>65–68,70</sup> as well as defences against the adaptive immune response<sup>79</sup>. There remain a number of unanswered questions regarding immune interactions between the trypanosome and its human host. One of the key examples of this is the question of how the invariant surface glycoproteins and other large non-VSGs on the surface are protected from immune recognition.

### MONO-ALLELIC EXPRESSION AND VSG CONTROL

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Both GPI-PLC and DOT1B interact with the VSG pathway. GPI-PLC has been confirmed in the literature to have a role in virulence which is not required for growth in HMI-11<sup>63,64</sup>. Finding this hit, therefore, increased our confidence in the results of our screen. GPI-PLC cleaves the GPI-anchors which attach the five million VSGs to the plasma membrane<sup>56,63</sup>. This releases them into the bloodstream during peak parasitaemia. The released VSGs then scavenge complement factors and antibodies. Potentially, this then allows a minority of the parasites to escape lysis by the immune system via the antibody-mediated complement activation pathway<sup>61,62</sup>. Therefore there is a clear hypothesis explaining why GPI-PLC influences parasitaemia in the murine model while remaining non-essential in the culture medium.

DOT1B has also been independently confirmed to have no essentiality *in vitro*<sup>125</sup>. It has also been shown that *dot1b*-null strains show a ten-fold drop in growth over 72 h in a serum system and substantially reduced viability in a plating assay that is not reflected in HMI-11 (Lucy Glover, personal communication). DOT1B is a histone



methytransferase which is required for accurate nuclear division during differentiation<sup>126</sup> and modulates the process of antigenic variation within the trypanosome. It has been shown that when the DOT1B gene is deleted, strict VSG silencing is no longer maintained and monoallelic transcription is compromised<sup>109</sup>. This creates an obvious survival burden on the trypanosome, as the strict integrity of its mono-allelic expression of the VSG genes is its greatest asset in avoiding antibody-mediated lysis by the host immune system. That being said, the three-day time period of the initial screen would not allow enough time for an adaptive immune response to be mounted. DOT1B may well play another role in trypanosome infectivity which is equally crucial in the early stages of the infection and the presence of such a role could also explain why a serum-specific phenotype was observed over three days by Lucy Glover in her work.

Although we have not tested GPI-PLC in SS1, we should be surprised to see a dramatic phenotype upon RNAi induction. This is because GPI-PLC appears to play a role directly in the parasite's defences against the immune system, and therefore the lack of GPI-PLC activity would not be expected to cause a growth defect in a system which, although closer to the *in vivo* nutrient balance, still lacks the host immune attack pathways. However, we were encouraged to see it in the *in vivo* screen results, where a specific phenotype was predictable based on previously published findings.

In an effort to understand and better treat sleeping sickness, bloodstream form *T. brucei* have been cultured in the laboratory setting since the 1970s. As detailed in the introduction to this work, these cultures originally required feeder layers of mammalian fibroblasts for continuous growth. The addition of various agents to IMDM allowed continuous culture without a feeder layer and formed HMI-11, now the standard routine cell culture medium for laboratory *T. brucei* strains<sup>95,96,98</sup>. However, while a convenient and comparatively economical option for routine lab work, HMI-11 has been shown to create a problem for drug discovery and the requisite accurate understanding of the dispensability of trypanosome genes<sup>99,103</sup>.

In order to address these problems, while seeking to minimise the use of animal models in our study, we have developed an *in vitro* system, SS1, which more closely matches the *in vivo* situation than the standard culture system. Our findings show that while it is more challenging than standard culture media, it is entirely possible to grow trypanosomes in this simple serum system providing both a copper chelator and a reducing agent are added.

Although SS1 is truer to the host situation than even carefully designed media such as Creek's Minimal Media, the limitations which it imposes on density do present challenges. Therefore, a more robust alternative would be useful. However, in my hands CMM proved ineffective.

In attempting to develop more robust growth, one experiment indicated that the addition of cysteine to our serum system allowed more robust parasite growth (data

not shown). This made sense within the historical context of *T. brucei* cell culture as cysteine was one of the first supplements added in transitioning to a system which allowed continuous growth without the presence of a human fibroblast feeder layer<sup>95</sup>. However, when I attempted to repeat this finding I saw initially high growth rates with subsequent declines and the death of the parasite cultures. Cysteine may still prove a valuable addition, but it may require increased levels of bathocuproinedisulfonic acid levels to buffer the increased cysteine and allow for healthy parasite growth.

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### PHOSPHATIDYLINOSITOL SIGNALLING

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In their recent work chemo-genetically characterising the inositol phosphate pathway, Cestari et al. demonstrated that most of the steps in the pathway are essential for growth and that some of those which are not essential for growth in the lab are still essential for infection. One of the genes in the latter category was Tb927.5.2690, the gene which encodes the second of *T. brucei*'s inositol-1(or 4)-monophosphatases. A null strain was created by Cestari et al. which showed no growth defect *in vitro*. However, infectivity was reduced. Parasitaemia only rose to detectable levels nine days post-infection and the resurgent parasites grew 2.5-fold slower than wild type. The reduction in infectivity and reduced growth confirms what I found using our serum-based culture system.

The precise molecular basis of Tb927.5.2690 dispensability *in vitro* is hard to determine because the product of the inositol monophosphatase enzyme that Tb927.5.2690 encodes, inositol, plays a key role in complex signalling processes. *Myo*inositol is one of the noteworthy constituents of HMI-11 which is overabundant when compared to average levels in human blood<sup>99</sup>. The HMI-11 recipe contains 1.6x the levels of

*myo*inositol found in human blood. Inositol is a key component which is required for glycosylphosphatidylinositol (GPI) synthesis<sup>127</sup>. GPI forms the anchor of the VSG and without the ability to synthesise it, the parasite would be unable to form the VSG coat and its defence against the host immune system would be dangerously compromised. Alternatively, in their earlier work, Cestari et al. noted that the inositol pathway appears to influence VSG expression at the telomeres<sup>128</sup>. If this pathway does regulate VSG expression then, again, a lack of *myo*inositol would compromise the anti-immune defences of the parasite.

The IMP knockdown parasites seem capable of surviving on the exogenous *myo*inositol which is in excess in the media. However, with the reduced *myo*inositol levels in SS1, the IMP knockdown parasites struggle to survive. In the host itself, with its complement and antibody mediated lysis defences under attack, the parasites would be under even stronger pressure to synthesise *myo*inositol.

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### FOLATE METABOLISM

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Folate is an essential nutrient for all organisms. It is required for the production and the maintenance of cells and for synthesising DNA and RNA. As such, it plays an especially important role in periods of high growth and rapid cell division. Insufficient folate will result in hindered DNA synthesis, cell division, RNA transcription and protein synthesis.

In humans, folate deficiency has been linked to infertility<sup>129</sup>, heart disease and stroke<sup>130</sup>, as well as being connected to a number of foetal defects in folate-depleted mothers<sup>131,132</sup>. Due to folate's huge public health importance, WHO placed it on their

list of the “minimum medicine needs for a basic health-care system... the most efficacious, safe and cost-effective medicines for priority conditions”<sup>133</sup>.

Folate is not generally found in the form of folic acid in the human body, with blood plasma levels being in the region of 0.02-0.08  $\mu\text{M}$  in healthy adults<sup>134,135</sup>. Instead, the biological functions of folate are performed by various derivatives of tetrahydrofolate. The most common of these (and the primary source of folate in the circulatory system of the trypanosome’s host) is levomefolic acid, or 5-methyltetrahydrofolate (5-MTHF)<sup>136</sup>. Within the cell, this can then be converted to tetrahydrofolate by various enzymes.

### 5-METE

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5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase (5-MetE) is one such enzyme which is unique to *T. brucei* and which was one of the hits we pursued as part of our screen. 5-MetE catalyses the transfer of a methyl group from 5-MTHF to L-homocysteine: forming tetrahydrofolate and methionine. As a result, it plays a key role in both folate metabolism and the *de novo* synthesis of methionine, both key processes for the parasite.

Why is there such a strong phenotypic difference between depletion of 5-MetE in our serum system/*in vivo* screen compared to HMI-11? One hypothesis is that, as methionine is seven times overabundant in the HMI-11 recipe compared to human blood<sup>99</sup>, the presence of this excess methionine prevents the parasite having to synthesise methionine *de novo*, thus removing the need for this pathway and making the gene dispensable. A second hypothesis is that without the ability to convert the readily available 5-MTHF into tetrahydrofolate, the parasite cannot generate enough

folate to sustain DNA synthesis and other processes which rely on sufficient folate levels for survival. In HMI-11 however, although there is a small source of 5-MTHF in the FBS, the main source is directly added folic acid – which is at a level three hundred times more abundant than the normal average in human blood. The parasite can, therefore, use this source of folate, bypassing the 5-MetE pathway in its entirety. These findings reflect what would be expected from the literature as compounds targeting folate metabolism have shown limited success in rich HMI-11 media while being extremely effective both *in vivo* and in folate-depleted media<sup>99,103</sup>. Folic acid can also outcompete classical antifolates for uptake into *T. brucei*<sup>137</sup>. Folic acid supplementation has also been shown to increase the risk of malaria to the extent that WHO reconsidered their recommendation of routine folate supplementation in affected areas<sup>138</sup>.

It seems likely that the answer lies in a combination of the hypotheses detailed above. Unfortunately, there may be other compensatory mechanisms in rich media that are yet unknown. We designed a rescue experiment using our simple serum system supplemented with various nutrients, predicting that a combination of folate and/or methionine and/or thymidine would rescue the RNAi phenotype and restore normal growth. The experiment appeared compromised due to a reduced phenotype, although initial indications are promising. Further work is required to confirm the effect of these compounds, perhaps with a gene knockout which is not as susceptible to adaptation.

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## IN VIVO VALIDATION

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No flask-based culture system can replicate the immune attack of the host. As a result of this limitation, we cannot completely discount any hits that do not validate in our simple serum system. However, we can rule out any that show a strong defect in HMI-11. We can also use this system to validate those hits which show a phenotypic difference between HMI-11 and *in vivo* studies either entirely or partially due to the difference in nutrient levels between the *in vivo* and *in vitro* environments (such as IMP or 5-MetE). As a result, we can be confident that the hits which we have validated using SS1 are true hits.

Demonstrating that our system is indeed an accurate representation of the *in vivo* environment will require animal studies in the future. However, the use of the SS1 system will minimise the number of mice that will be required and reduce the costs and complexity of validating the results from this and future screens.

## FUTURE WORK

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### PROTEIN BIOCHEMISTRY

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Utilisation of existing tagging vectors will allow localisation of the proteins encoded by our genes of interest and verification of knockdown<sup>119</sup>. We should expect to see proteins expressed on the cell surface and in the flagellar pocket, as these are most likely to be involved in host:parasite interactions. We should also expect several proteins to be secreted; and there may also be cytosolic proteins which are involved in metabolic processes specific to the *in vivo* situation. Localisation of these proteins using GFP or c-myc tags will give more information about their potential functions and guide our analysis. Data from ongoing whole-genome tagging approaches such as TrypTag<sup>121–123</sup> will also be used to guide our approaches, although these may prove to be of limited direct relevance as, to date, high-throughput gene tagging is only possible in procyclic parasites. See Appendix 1, Table 6 for data available on TrypTag to date.

### CHARACTERISING HOST-PARASITE INTERACTIONS

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The initial workflow described above will be followed to characterise each new hit from the screen which we attempt to validate. Once it has been confirmed that depletion of the gene product causes a phenotype in SS1 which is not reflected in HMI-11 then the process would depend on the suspected function of the gene product.

If it was suspected from the bioinformatics data that the gene product had a role in metabolism in the host, then we would pursue this by metabolic studies. Using a minimal medium such as SS1 or CMM and comparing parasite survival rates at culture



media levels of these specific metabolites or more physiological concentrations would help to validate the suspected purpose of the genes.

If the genes were suspected to be involved in immune evasion in the host, then this would require the use of immunocompromised mice to verify the suspected function. However, other avenues would be investigated before committing to animal studies. If these studies prove appropriate at a later stage then a cohort of five control and five mutant mice would be used for each experiment.

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### CAS9 AND GENOME EDITING

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The CRISPR-Cas9 system is based on the 'adaptive immune defences' of bacteria and archaea which exist to protect them against invading nucleic elements such as those from viruses<sup>139</sup> or plasmids. RNA-guided nucleases such as Cas9 are reviewed in more detail here<sup>140</sup>.

The CRISPR-Cas system would be ideal for validation of our genetic screen. As our hits are expected to be dispensable *in vitro* but essential *in vivo*, the generation of knockouts is possible, unlike for genes essential both *in vitro* and *in vivo*. Although it is possible to use RNAi techniques to knock down the genes of interest, and this is being used in the initial validation stage, knockout of the gene is much cleaner than a partial knockdown and guarantees complete removal of the gene. We do not yet have a robust system established for precise gene knockout using Cas9 in *T. brucei*, but this work is ongoing.

## APPENDIX 1: RNAI SCREEN HITS

TABLE 5: LIST OF RNAI SCREEN HITS

Gene ID (Tb927.)	Gene Product	RNAi Fold Change	# TM	SPP	Putative Category
1.3400	hypothetical protein, conserved	4.1	0	null	Unknown
1.4220	hypothetical protein, conserved	21.8	0	null	RNA [a]
1.570	DOT1B	18.6	0	null	Surface, Cell Cycle
2.3080	fatty acid desaturase, putative oleate desaturase, putative	4.4	6	null	Metabolism
2.3320	65 kDa ISG	5.1	1	0.62	Surface
2.6000	GPI-PLC	4.7	0	null	Surface
3.2640	hypothetical protein, conserved	6.5	0	null	Unknown
4.1030	hypothetical protein, conserved	5.2	0	null	Unknown
4.2750	hypothetical protein, conserved	6.9	0	null	Unknown
4.3620	protein phosphatase 1, putative	5.0	0	null	Protein Modification
4.3630	protein phosphatase 1, putative	10.5	0	null	Protein Modification
4.4070	mevalonate kinase, putative	4.6	0	null	Metabolism
4.4340	hypothetical protein, conserved	5.0	0	null	Unknown
4.4770	hypothetical protein	16.6	1	0.28	Unknown
5.2580	hypothetical protein, conserved	11.1	1	null	Gene expression [b]
5.2690	inositol-1(or 4)- monophosphatase 1, putative	10.4	0	null	Metabolism, Signalling
5.3000	hypothetical protein, conserved	6.7	1	null	Metabolism [c]
6.1640	single strand-specific nuclease, putative	11.2	0	0.98	DNA repair, Cell Cycle
6.2140	hypothetical protein, conserved	28.1	0	0.62	Transport, RNA [d]
6.2820	hypothetical protein, conserved	13.9	0	null	Unknown
6.2860	hypothetical protein, conserved	6.2	0	null	Unknown
6.4220	mitogen-activated protein kinase 5	5.6	0	null	Stress Response
6.4320	hypothetical protein, conserved	13.4	1	0.11	Unknown
7.4090	serine/threonine-protein kinase, putative	6.4	0	null	Protein Modification
7.6700	hypothetical protein	14.2	0	null	Unknown
7.6820	hypothetical protein, conserved	7.4	0	null	Unknown
8.2230	Checkpoint protein HUS1, putative	INF	0	null	DNA repair, Stress Response, Cell Cycle
8.2610	5-MetE, putative	6.4	0	null	Metabolism
8.6230	hypothetical protein, conserved	14.9	0	null	Unknown
9.9030	hypothetical protein, conserved	6.0	0	null	Metabolism [e]
10.12720	hypothetical protein, conserved	12.2	0	null	Unknown
10.13350	hypothetical protein, conserved	4.6	0	null	Unknown
10.14700	hypothetical protein, conserved	4.4	0	null	Unknown
10.14930	zinc finger protein family member, putative	739.5	0	null	RNA, Protein Modification

<b>10.3880</b>	hypothetical protein, conserved	5.9	1	null	Unknown
<b>10.4260</b>	hypothetical protein, conserved	7.3	10	0.90	Transport [f]
<b>10.5670</b>	N-acetyltransferase subunit Nat1, putative	4.0	0	null	Metabolism
<b>10.6610</b>	chaperone protein DNAj, putative	5.2	1	null	Stress response
<b>10.8730</b>	hypothetical protein, conserved	5.8	0	0.98	Metabolism [g]
<b>11.13120</b>	hypothetical protein, conserved	6.6	0	null	Protein Modification [h]
<b>11.6560</b>	hypothetical protein, conserved	40.8	0	null	Unknown
<b>11.7380</b>	glycerol-3-phosphate dehydrogenase (FAD-dependent), mitochondrial	5.7	0	0.60	Metabolism
<b>11.8400</b>	Mitochondrial RNA processing endonuclease 1 (mRPN1)	6.7	0	null	RNA
<b>11.890</b>	hypothetical protein, conserved	4.5	0	1.00	Unknown
<b>11.9070</b>	palmitoyl acyltransferase 4, putative	4.1	4	null	Protein Modification

List of hits from the RNAi *in vivo* screen. The three genes chosen for this project are highlighted in green. # TM refers to the number of transmembrane domains (by sequence similarity to existing known domains). SPP refers to the probability of the sequence containing a signal peptide (by sequence similarity to known domains). Putative categories determined by reference to similarity to existing protein families.

CDD: Conserved domain database<sup>115,116</sup>, BLAST: Basic Local Alignment Search Tool<sup>113</sup>

[a] CDD: RNase\_H2-B superfamily

[b] CDD: PHA03307 family of transcriptional regulators

[c] BLAST: thiopurine S-MTase; CCD: AdoMet\_MTases superfamily

[d] BLAST: decarboxylase; CCD: FAA\_hydrolase superfamily

[e] CDD: Glycosyltransferase family A

[f] BLAST/CDD: LMBR family

[g] BLAST: ubiquinone biosynthesis protein, ABC1 family/Phosphotransferase enzyme family; CDD: ABC1 kinase-like proteins, ABC1, Ubiquinone biosynthetic protein UbiB

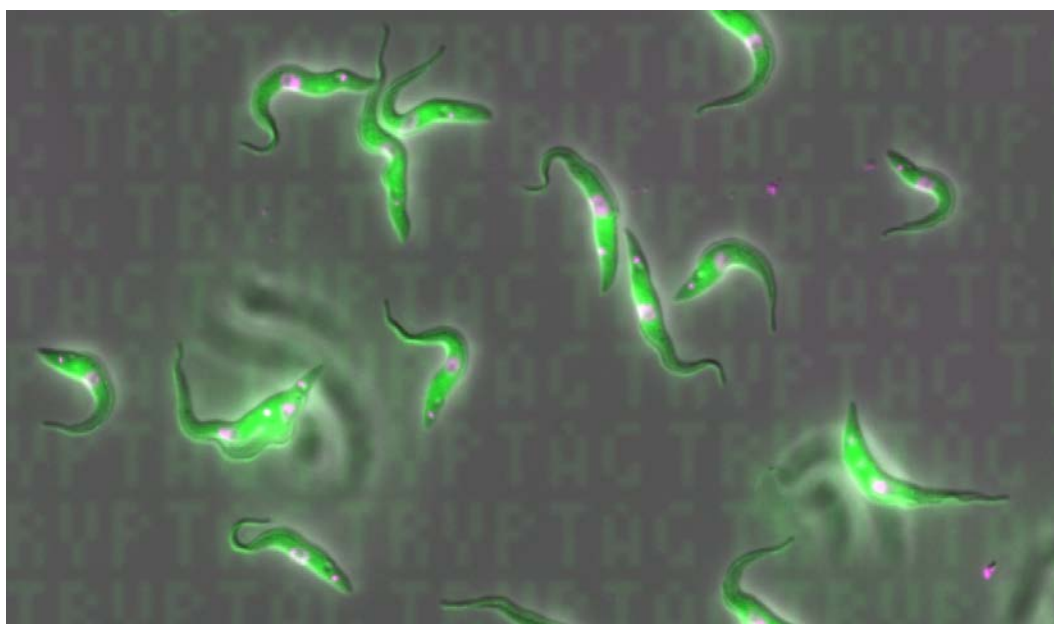
[h] CDD: PUB superfamily

TABLE 6: TRYPTAG DATA FOR SCREEN HITS

Gene ID (Tb927.)	Gene Product	Tryptag Localisation
1.4220	hypothetical protein, conserved	Mitochondrion
1.570	DOT1B	Nuclear lumen (Cell cycle Dependent)
4.1030	hypothetical protein, conserved	nuclear lumen, cytoplasm
4.2750	hypothetical protein, conserved	cytoplasm (reticulated)
4.3620	protein phosphatase 1, putative	Cytoplasm (reticulated), axoneme (<10%, strong), cytoplasm (points, 25%)
4.4070	mevalonate kinase, putative	Glycosome (75%)
5.2690	inositol-1(or 4)-monophosphatase 1, putative	Cytoplasm, flagellar cytoplasm, nuclear lumen
6.2860	hypothetical protein, conserved	cytoplasm (points)
10.12720	hypothetical protein, conserved	hook complex
10.14700	hypothetical protein, conserved	Cytoplasm, nucleoplasm, nucleolus (strong)
10.14930	zinc finger protein family member, putative	acidocalcisome
10.3880	hypothetical protein, conserved	cytoplasm (points, reticulated)
10.5670	N-acetyltransferase subunit Nat1, putative	cytoplasm
11.6560	hypothetical protein, conserved	basal body, pro-basal body
11.8400	Mitochondrial RNA processing endonuclease 1 (mRPN1)	cytoplasm (points)
11.890	hypothetical protein, conserved	cytoplasm (weak), endocytic

TrypTag localisation data. Shows data, where available from TrypTag<sup>121–123</sup>, regarding the localisation of hits from the screen. Genes from Table 5 above which are not present here have not been reported by the TrypTag project team to date.

FIGURE 12: TRYPTAG IMAGE FOR IMP



TrypTag image for IMP. Green = N-Terminally tagged IMP, Magenta = Dapi.

## APPENDIX 2: AUTONOMOUSLY REPLICATING SEQUENCE SCREEN

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### INTRODUCTION

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Study of *Trypanosoma brucei* is hampered by a lack of genetic tools. One such missing tool is access to autonomously replicating sequences for episomal replication. One study found a few autonomously replicating plasmids in procyclic form *T. brucei*. These could exist as highly stable single-copy episomes within the parasite<sup>141</sup> and proved useful in characterising elements of trypanosomal DNA replication<sup>142</sup>. During my rotation, I aimed to create a vector which would enable whole genome screening in bloodstream form trypanosomes in order to find and characterise these sequences.

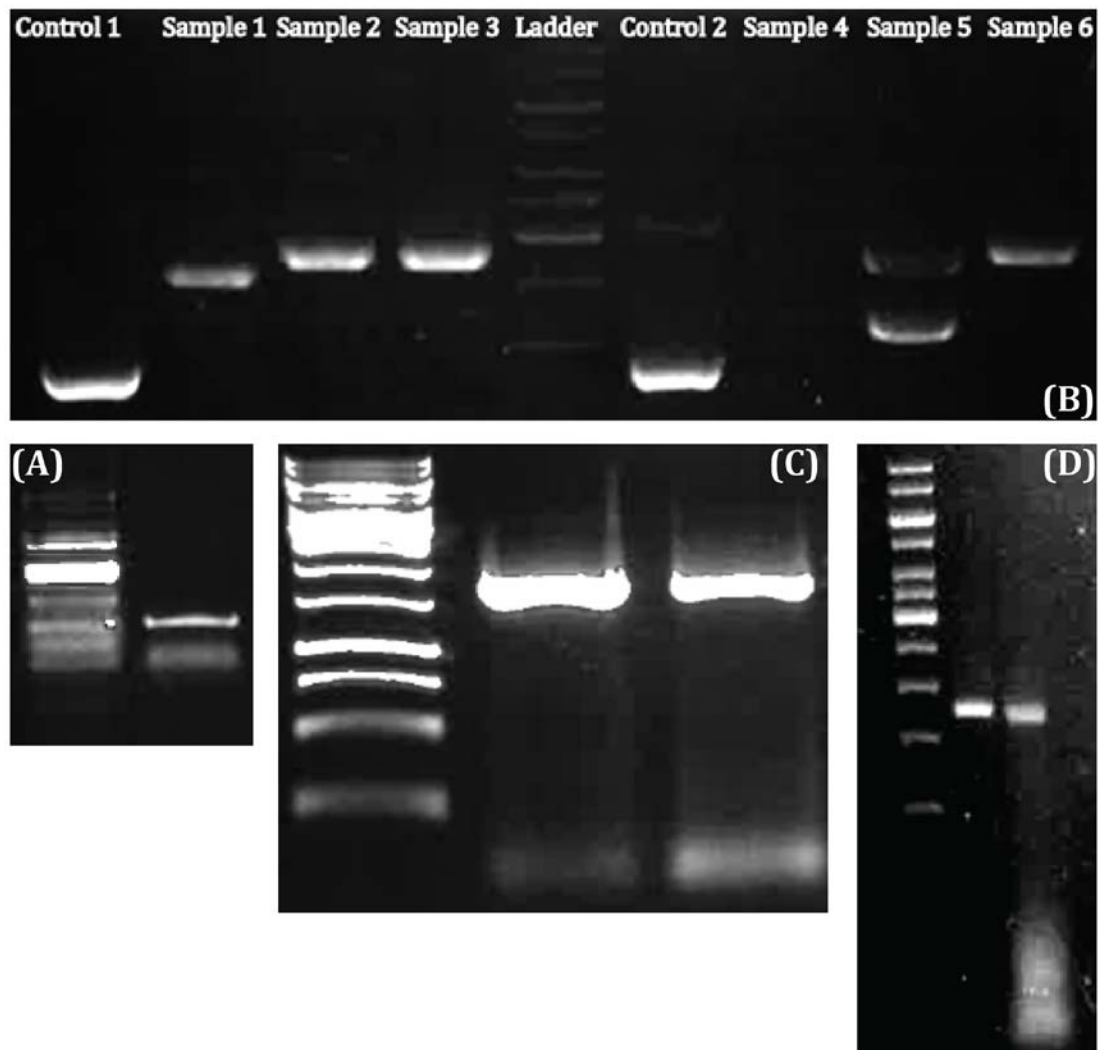
### METHODS AND RESULTS

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To accomplish this goal, the LacZ stuffer fragment from pRPa<sup>ISL</sup> was amplified and digested with SacI / EcoRV. It was then cloned into SacI / NaeI digested pGEM-T easy. The NPT resistance cassette was amplified from p5'neo5' and digested with EcoRI and ligated to a T7 terminator with sticky ends for EcoRI and XbaI before being digested with SacII with the aim of cloning it into SacII / XbaI digested vector.

Synthesising the T7 terminator within the NPT primer proved unworkable. Instead, the T7 terminator was inserted as a pair of annealed primers which were ligated to the NPT PCR product before it was inserted into the vector. I successfully amplified the LacZ stuffer fragment (Figure 13A) and completed cloning step one (Figure 13B). I also successfully amplified the NPT cassette (Figure 13C) and ligated it to the T7 terminator (Figure 13D). However, the final ligations of the NPT cassette to the vector were unsuccessful.

FIGURE 13: CLONING RESULTS



Cloning results. (A) Left to right – Ladder and LacZ PCR product; (B) results of LacZ-pGEM-T Easy ligation, samples 2, 3 and 6 showing expected band (1,100bp), sequenced to verify; (C) Left to right – Ladder, NPT PCR product, NPT PCR product; (D) Left to right – Ladder, NPT, NPT ligated to T7 terminator

## CONCLUSION

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We aimed to create a vector which would allow us to screen the trypanosome genome for autonomously replicating sequences in order to further characterise the nature of these sites and hopefully create new genetic tools. The vector was not completed but several important obstacles were surmounted and the first stage of the cloning process was successful. The PCR amplification of the NPT cassette, a major bottleneck in the cloning strategy, was successfully optimised. I also successfully ligated the NPT cassette to the T7 terminator and verified this by running the ligation on an agarose gel. However, the ligation of this to the vector created in the first cloning step proved challenging. Subsequently, the decision was taken to synthesise this vector instead of attempting to construct it via enzyme digests.

## APPENDIX 3: QUORUM SENSING

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### INTRODUCTION

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Quorum sensing is the mechanism by which organisms communicate at high cell density and is used by many species to control differentiation, forming part of their response to the environment. Quorum sensing normally involves the production of small, extracellular signalling molecules called autoinducers (AIs). AIs are then detected by other members of the species, triggering the desired response. Quorum sensing has been most closely studied in bacteria, where it has been linked to many processes such as bioluminescence, sporulation, antibiotic production and virulence factor secretion, to name a few<sup>143</sup>. A feed forward loop is normally created by the reception of the AIs stimulating their production – thus enabling a rapid and synchronous response. Although the molecular details of the AIs and the feed forward loop vary greatly from system to system, the basic principles are the same across many organisms.

Compared to other species, quorum sensing systems in trypanosomes are quite poorly understood. What is known from research over the last two decades is that differentiation and density sensing are controlled via a cyclic AMP-linked stumpy induction factor (SIF)<sup>144</sup>, which is thought to be soluble, heat stable and of low molecular weight.

A recent genome-wide RNA interference library screen looking for resistance to the Phosphodiesterase Inhibitor CpdA identified the first downstream cAMP effector proteins found in *T. brucei*<sup>145</sup>. A second screen was used to identify the signalling



components driving stumpy formation in order to shed some light on genes which may be involved in quorum sensing mechanisms in trypanosomes<sup>146</sup>. This latter study looked for genes which, when knocked down, made the trypanosomes unresponsive to cell-permeable analogues of cyclic AMP. It provided an initial identification of some of the molecules and processes required for *T. brucei* differentiation and opened up some new potential targets for future drug discovery.

Prior to these screens, a RIT-seq screen was carried out by our lab which showed a gene with a distinct gain of fitness phenotype at high density<sup>107</sup>. This was confirmed by a subsequent unpublished screen carried out by Sam Alsford. The hits from this screen did not overlap with the data sets mentioned above and five of the nine key hits had a connection to calcium or the calcium-binding messenger protein calmodulin, which indicates a role in a separate, calcium-dependent pathway.

I aimed to investigate this gene and to understand the relation of the gene and the potential wider calcium-mediated density based signalling mechanism to the classical SIF pathway.

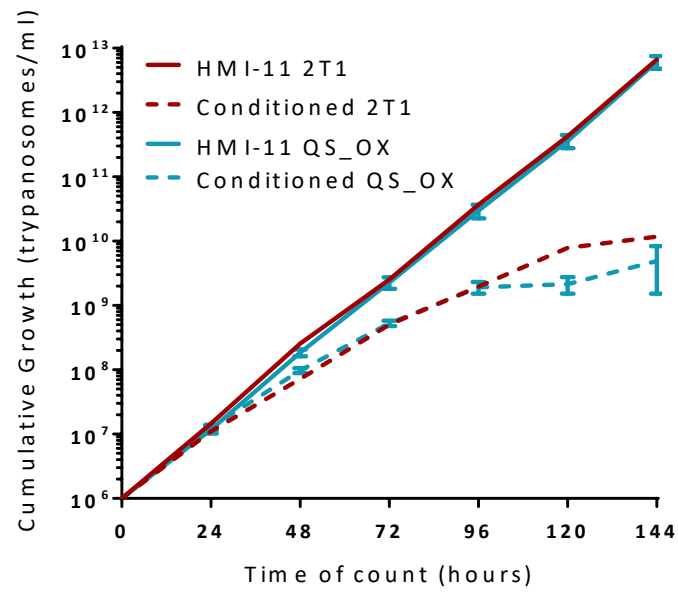
## METHODS AND RESULTS

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Density sensing proteins were discovered and confirmed by Sam Alsford using the RNAi library screening methods previously described<sup>107</sup>. The gene was cloned using high-fidelity PCR and cloned into pRPa<sup>iSL</sup> to generate an untagged overexpression construct<sup>117</sup>. For tagging at the native loci, an N-terminal fragment was amplified and cloned into pNAT<sup>TAGx 117</sup>.

For the growth curve, 2T1 cells were seeded at  $10^5$  cells per ml every day. From this culture, a feeder flask was seeded daily at  $5 \times 10^5$  cells per ml. After 24 h this feeder flask was spun down and the media filtered to provide conditioned media for the growth curve in order to boost the levels of the signal received (or mediated) by the product of this quorum sensing gene. Two independent biological clones of the over-expresser strain were split and seeded at  $10^5$  cells/ml in this media. The cells were counted every 24 hours using a haemocytometer and diluted as appropriate. Induction was by tetracycline at  $1 \mu\text{g/ml}$ . After five days of splitting these cells in this conditioned medium a difference was observed between the over-expresser and the 2T1 control. One of the two independent biological clones appears to have adapted as it grew out on the last day, skewing the results, but the indication is that overexpression of Tb927.8.6870 makes the parasite more sensitive to conditioned media (Figure 14).

FIGURE 14: OVER-EXRESSER SHOWS SLIGHTLY INCREASED DEFECT IN  
CONDITIONED MEDIA



Growth curve using conditioned media and an over-expression cell line to probe quorum sensing mechanisms in *T. brucei*. Error bars show standard deviation from the mean between two independently-generated biological clones.

## CONCLUSION

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Many organisms, including the trypanosomes, use density-sensing mechanisms to communicate and synchronise beneficial behaviours. As a blood-borne parasite with a complex life cycle requiring adaptation between stages, this is particularly important for *T. brucei*.

Our unpublished RIT-seq screen (Sam Alsford) showed multiple hits which conferred a gain of fitness at high density. Over 70% of these reads fell on a single gene. The hits, however, did not overlap with the pre-existing data sets, indicating that this protein is not part of the canonical SIF pathway, although the pathways may well intersect. This is backed up by the number of hits in the screen which had a connection to calcium or calmodulin.

One of the RNAi target fragments that were picked out of the screen actually hits the gene next to Tb927.8.6870. Interestingly, based on homology, this gene is thought to be a dauer formation protein. Dauer (German for endurance) proteins are known in *C. elegans* to be involved in the progression to a quiescent state that allows the worm to survive harsh conditions. It is tempting to hypothesise that this protein is an effector which is triggered by the quorum sensor we have discovered.

Unfortunately, Tb927.8.6870 knockout strains made by Sam Alsford did not replicate the phenotype seen in the screen. This could be due to adaptation and we are making inducible RNAi strains to address this problem. We hope to repeat our experiment using the null strains and these new RNAi strains in addition to the over-expresser.

Calcium sensors such as aequorin and pericam would also help to probe the link to calcium.

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